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<b>(21) International Application Number:</b> PCT/GB90/01046 <b>(22) International Filing Date:</b> 6 July 1990 (06.07.90)  <b>(30) Priority data:</b> 8915512.1                      6 July 1989 (06.07.89)                      GB  <b>(71) Applicant (for all designated States except US):</b> SECRETARY OF STATE FOR HEALTH IN HER BRITANNIC MAJESTY'S GOVERNMENT OF THE UNITED KINGDOM OF GREAT BRITAIN AND NORTHERN IRELAND [GB/GB]; Room 203, 14 Russell Square, London WC1B 5EP (GB).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> ROCKS, Bernard, Francis [GB/GB]; 3 Cliff Road, Roedean, Brighton BN2 5RD (GB). BAILEY, Michael, Philip [GB/GB]; 42 Lewes Road, New Haven, East Sussex BN9 9RY (GB). BERTRAM, Vanessa, M., R. [GB/GB]; Old Orchard, Little London, Heathfield, East Sussex TN21 OBA (GB).		<b>(74) Agent:</b> BECKHAM, Robert, William; Ministry of Defence, Patents 1A, Room 2014, Empress State Building, Lillie Road, London SW6 1TR (GB).  <b>(81) Designated States:</b> AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CM (OAPI patent), DE*, DE (European patent)*, DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US.  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> SILVER ENHANCED GOLD-LABELLED IMMUNO ASSAY METHOD		
<b>(57) Abstract</b>  A silver enhanced gold-labelled immunogold assay (SEGLISA) capable of determining levels of target analytes such as antigens, antibodies and drugs in physiological fluids, including whole blood samples, is disclosed. The assay is particularly suited for the determination of the presence and/or amount of HIV and Rubella antibodies and provides a permanent result of less equivocal nature than prior ELISA assays. Assay of samples provided by spotting whole blood onto blotting paper by the technique opens up the possibility of submitting samples by post.		

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SILVER ENHANCED GOLD-LABELLED IMMUNO ASSAY METHOD.

The present invention relates to a method of assaying a fluid for analytes capable of undergoing specific binding reactions, such analytes being, for example, specific antibodies or antigens, said antigens being for example proteins, viruses, hormones, drugs, nucleic acids and polysaccharides.

The present invention further provides a method of determining, quantitatively or qualitatively, the presence and/or amount of specific analytes, particularly antibodies, in physiological fluids, particularly whole blood and blood products. Assay for specific antibodies in physiological fluids, whether of IgG, IgM, IgD or IgA classes, is the principle method of detecting infection by antigens, particularly of bacterial or viral infections, to which the antibodies are specific. The use of enzyme linked immunosorbent assay (ELISA) methods for the detection of antibodies is well known.

Generally, in an ELISA method, the antibody or antigen being assayed is captured by a respective antigen or antibody immobilised on a substrate and is then exposed to a secondary antibody labelled with an enzyme. The secondary antibody binds to the captured antibody or antigen such that its presence or amount may be assayed as related to bound enzyme activity, usually by an enzymic reaction producing a colour change.

Such methods are of particular importance at present due to the widespread screening necessitated by the discovery of human immunodeficiency virus (HIV), previously referred to as human T-cell lymphotropic virus type 3 (HTLV-III) or lymphadenopathy associated virus (LAV), and its role in acquired immune deficiency

syndrome (AIDS).

Tests for detecting infection by HIV are of particular value in studying the spread of the epidemic, as an aid to the diagnosis of AIDS and in screening blood to avoid the use of donations from infected people. Since HIV may be transmitted by blood transfusion and by blood products (eg: Factor VIII as used for treatment of haemophilia) the testing of all blood donations has become a necessity. Screening tests currently used rely upon detection of specific antibodies in bodily fluids, especially in blood.

In addition to the ELISA method other assays have become available including the use of immunofluoresence, radioimmuno-assay and Western blotting. Compared to these formats ELISA has the advantage of being simpler and less expensive but several problems are still evident. Use of enzyme labels requires an extra step in which the tests, usually carried out in microtitre wells, are incubated with enzyme substrate reagent in order to produce a coloured product. The colour reaction is allowed to proceed for a set time and the reaction is stopped using a strong mineral acid before reading the colour visually or photo-metrically. This step is prone to errors and adds significantly to the time needed to produce results. ELISA also suffers from the bulky, labile nature of the enzyme label, false results with whole blood and susceptibility to inhibition and denaturation.

More recently assays have appeared which replace enzyme label on the secondary antibody with conjugated gold label, these being known as 'immunogold assays'. The use of gold labelled secondary antibodies to the antibodies or antigens under assay allows

visual identification of the antigen-antibody complex as a pink spot.

Whereas ELISA generally requires sophisticated equipment to measure substrate and product levels the immunogold test does not. Unlike the several hours often required to complete the ELISA, due to the substrate incubation step, the immunogold assay can provide results in as little as 10 minutes and thus prove of great assistance in the preparation of products such as Factor VIII where speed of extraction from freshly donated batches of blood is essential. Such immunogold assays are in use for example in the DuPont HIVCHEK (registered trade mark) and the Organon Teknika SPIA. The gold conjugate is relatively inexpensive, does not have the lability of enzyme labels and fewer manipulations are required over the ELISA assay.

The immunogold assays are not without problems of their own. The gold label provides a pink colouration as a positive result that is often very difficult to see. As ten percent of males have some colour blindness in the region of light concerned problems may arise in detection without the technician being aware of it. Furthermore, the test, as with ELISA, requires the use of serum or protein free fluids such as urine to function correctly.

Gold labelled antibodies have also been used in histology to stain entire cells and tissues so as to allow their structures to be seen under the microscope. In histology a technique for enhancing the gold stain by using the bound gold particles to catalyse local nucleation of silver particles from a solution of a silver salt has been developed which results in a much darker staining (see Danscher. G, Histochemistry 71. 81. (1981);

Holgate. C et al, J. Histochem, Cytochem 31 938 (1983). The technique of silver enhancement (known in the art as 'immunogold silver staining' or 'IGSS') has problems such as self nucleation of silver which leads to deposition in areas other than those labelled with gold and the requirement that specific protocol is needed to achieve optimum results. The use of such silver enhancement in immunogold assays has not been proposed or investigated. It is an object of the present invention to provide visually enhanced immunogold assays; permanent record immunogold assays and the capability to perform a reliable immunogold assay on a whole blood sample; the latter being unassayable by present ELISA techniques. Other objects and advantages of the present invention will be apparent to those skilled in the art on reference to the following specification and Examples.

The present invention provides a method for visually, and/or photometrically and/or colorimetrically enhancing immunogold assays comprising exposing at least partially completed immunogold assay tests to a solution comprising silver ions whereby silver nucleates out onto at least a portion of any gold on said assay and then relating the presence and/or amount of nucleated silver to the presence and/or amount of the assay target analyte.

The present invention thus provides a first non-competitive enhanced immunogold assay option for the determination of the presence and/or quantity of a target analyte in a fluid comprising:

- (a) immobilising onto a solid substrate a specific binding agent with which the target analyte will combine specifically

(b) exposing the immobilised specific binding agent to a sample of fluid to be assayed

(c) exposing the immobilised specific binding agent from step (b) to a secondary binding reagent capable of binding to the specifically combined target analyte and to which colloidal gold particles have been bound

(d) exposing the immobilised specific binding agent from step (c) to a solution containing silver ions whereby the silver nucleates onto at least a portion of the gold bound to any bound secondary binding agent and

(e) relating the presence and/or amount of nucleated silver to the presence and/or amount of target analyte in the fluid.

There is further provided a second, competitive, option for the performance of an enhanced immunogold assay of the invention, said option comprising:

(a) immobilising onto a solid substrate a specific binding agent with which the target analyte will combine specifically

(b) exposing the immobilised binding agent to a mixture of the fluid to be assayed and a solution containing a quantity of target analyte, or an analogue thereof which will bind to said specific binding agent, to which colloidal gold particles have been bound

(c) exposing the immobilised binding agent from step (b) to a solution of silver ions whereby silver nucleates onto at least a portion of any gold bound to said binding agent via any labelled target analyte or analogue thereof and

(d) relating the presence and/or amount of nucleated silver to the presence and/or amount of target analyte in the fluid to be



tested.

A still further, competitive, option for the performance of an enhanced immunogold assay of the invention is provided comprising:

(a) immobilising onto a solid substrate a target analyte with which a specific binding agent will combine specifically

(b) exposing the immobilised target analyte to a mixture of the fluid to be assayed and a specific binding agent with which the target analyte will combine specifically

(c) exposing the immobilised target analyte from step (b) to a secondary binding agent capable of binding to the combined specific binding agent and to which colloidal gold particles have been bound

(d) exposing the immobilised reference target analyte from step (c) to a solution containing silver ions whereby silver nucleates onto at least a portion of the gold bound to any bound secondary binding agent and

(e) relating the presence and/or amount of nucleated silver to the presence and/or amount of target analyte in the fluid to be tested.

In each of the options the presence of the target analyte in the test fluid is determined by the presence or lack of silver deposit. In the first, non-competitive, option a positive result is provided by the presence of a silver deposit whereas the two competitive options show a reduced or no deposit as indicative of a positive result indicating that the labelled material has been prevented from binding by competition from analyte in the test fluid. The amount of analyte may be determined from the degree



of deposit, for example visually, colorimetrically or photo-metrically. Use of standard curves or colour charts for known analyte concentration absorbance values for the immobilised surface provides a convenient yet accurate measure of the amount of analyte in the test fluid. The invention is expected to be useful in the enhancement of all types of immunoassays in which colloidal gold is used as a labelling agent and other options will occur to a man skilled in the art of binding assays.

The invention further provides kits for the performance of the method of the invention, said kits comprising substrate having specific binding reagent or target analyte immobilised thereon, a solution comprising silver ions, and either gold labelled secondary binding reagent or gold labelled target antibody.

The methods and kits of the present invention provide silver enhanced immunogold assays which at least partially overcomes the problems associated with the ELISA and immunogold assays previously described and provide a still higher degree of sensitivity with a absorbance improvement of 100 times being routinely obtained.

By use of a silver ion containing enhancer solution the gold is completely visualised to provide a dark brown to dense black deposit of metallic silver on the gold which may be produced in an amount proportional to the amount of gold labelled reagent bound to the solid-phase specific binding reagent. In the first option when the sample contains no target analyte then the secondary binding reagent gold conjugate cannot bind and no colour is produced thus giving a simple visual indication that no target analyte is present; the competitive assays giving a

darkened negative. Results may alternatively be read visually using a conventional colorimetric plate reader with no filters being necessary.

As the silver layer is permanent the dried plates may be kept for future reading or checking. All the reagents are stable and the silver enhancement takes place under ambient conditions. An important advantage of the present method is its ability to assay whole blood as opposed to the prior assay's essential use of serum, plasma or non-blood liquid.

The method of the invention may be used to detect any target analyte in any aqueous fluid. Typical analytes include specific proteins, viruses, hormones, drugs, nucleic acids and polysaccharides; specifically antibodies, eg: IgD, IgG, IgM or IgA immunoglobulins to HTLV-I, HIV, Hepatitis A, B and non A/non B, Rubella, Measles, Human Parvovirus B19, Mumps, Chicken Pox or Leukemia; human and animal hormones, eg: human growth hormone (hGH), human chorionic gonadotropin (hCG); drugs, eg: paracetamol or theophylline ; marker nucleic acids, eg; as for genetic fingerprinting analysis markers; polysaccharides such as cell surface antigens for HLA tissue typing and bacterial cell wall material; proteins such as carcinoembryonic antigen (CEA) or alpha-fetoprotein. Typical aqueous fluids include physiological fluids such as human or animal whole blood, blood serum, blood plasma, semen, tears, urine, sweat, saliva, cerebro-spinal fluid, vaginal secretions; in-vitro fluids used in research or environmental fluids such as aqueous liquids suspected of being contaminated by the analyte.

When used with serum this may be used diluted or undiluted.

Whole blood may also be used diluted but is preferably used diluted, eg: in the range 1:1 to 1:10, but dilution up to about 1:5000 is still found to yield useful results. A suitable diluent is a standard specimen diluent, eg: phosphate buffer saline.

In the assay of certain analytes, such as HIV antibody, there may be a degree of social sensitivity and the method of the invention advantageously enables assay of fluid specimens in the form of samples blotted onto an absorbant material such as filter paper which can be allowed to dry and may be extracted using a suitable aqueous medium such as phosphate buffer saline; the dried sample may then be assayed as above. As little as 20 microlitres of a fluid such as whole blood need be blotted onto the absorbant material in this embodiment and extraction into typically 20 times this volume of aqueous medium provides a suitable extract for assay.

The solid substrate may be any material capable of having the specific binding reagent immobilised thereon; a preferred material being plastics or cellulose nitrate. The substrate may conveniently be of rod, bead or plate form. Most preferably the substrate is a microtitre plate and the specific binding reagent is immobilised into one or more of the wells on its upper surface using techniques known in the art, such as those employed in conventional ELISA assay substrate preparation.

The specific binding reagent may be any material to which the analyte will bind specifically. It may, for example, be whole cells or tissue, cell fragments or protein, including protein which has been harvested from, or made by recombinant means from,

an organism for which the infection thereby is being assayed for. In the case of HIV the specific binding reagent may be the antigens which are commercially available and substrates already having such an antigen immobilised thereon are also commercially available, eg: as antigen coated microwell strips available from DuPont, as part of the DuPont HTLV-III/LAV ELISA Kit. When such commercially available substrates and immobilised antigen are used step (a) may be avoided in the practical performance of the assay.

The gold labelled secondary binding reagents may comprise any antibody binding to the target analyte and having colloidal gold particles conjugated to it. Preferably the gold particles are 10nm or less in size, 5 nm or less being the most effective. The exact nature of the secondary binding reagent may of course depend on the nature of the target antibody under investigation and suitable secondary antibodies will be apparent to those skilled in the art. Methods of conjugating colloidal gold particles to these secondary antibodies are known in the art and gold labelled secondary binding reagents are commercially available. These may be used in the method of the invention but preferably in a diluted state. A preferred concentration of gold labelled secondary binding reagent is that achieved by diluting the commercially available AUROPROBE LM GaHu (Mercia Diagnostics Ltd) in the range of 1:10 to 1:50; the 1:50 dilution proving to be most effective, Preferably other types of gold labelled reagent should be diluted to achieve this level of concentration.

A wide range of anti-human, (eg: anti- $\mu$  and  $\gamma$ ), anti-rat, anti-mouse, anti-guinea pig and anti-rabbit antibodies which may

be used as secondary binding reagents already labelled with gold are commercially available, eg: those available from Janssen Life Science Products under the registered trade mark AUROPROBE. For the purpose of testing human antibodies to most infections, eg HIV, the anti- $\gamma$  agents which bind specifically to IgG are most useful as is Protein-A, a protein widely used for the detection of antibody molecules which is also available commercially bound to gold.

The silver ions in the solution used in step (d) may be supplied by any ionisable silver compound provided that the counter ion does not otherwise interfere with the assay. Preferred silver compounds are salts of organic acids, especially silver citrate or silver lactate, but any soluble inorganic silver salt, such as silver nitrate, may be used. The concentration of silver ion in the solution may vary greatly but is preferably in the range 0.1 to 1000 mM, more preferably from 1 to 100 mM, particularly from 2 to 20 mM. It is preferred to include a reducing agent, eg: an amino or hydroxybenzene such as hydroquinone, and a retardant for prevention of nucleation occurring too quickly, eg: gum acasia to the silver ion containing solution of step (d). The silver deposit may further be fixed onto the well using a suitable solution such as sodium thiosulphate although this is usually unnecessary. The preferred ingredients are selected to produce a light-stable assay relatively insensitive to water impurities with a large safety margin between adequate silver ion concentrations for the sensitivity of the assay and silver concentrations at which self-nucleation leads to unacceptably high background deposition.

Suitable silver ion containing solutions for use in step (d) are commercially available, eg: for use in the IGSS methods described above as sold by Janssen under the registered trade mark IntenSE\*II or by Sigma Chemical Co under the mark SE-100.

A typical protocol for the first non-competitive option for performing the assay method of the invention is exemplified as applied to HIV as follows. Steps (a) and (b) may be carried out in a manner identical to that used in the known ELISA methods, except that whole blood, preferably diluted with phosphate buffered saline (PBS) may be used as the fluid. When the fluid is serum it is preferred to expose the immobilised specific binding reagent, ie: the antigen, first to PBS containing goat serum for about two minutes to prevent non-specific binding at lower concentrations of goat serum than would otherwise be used. The method is found to work at serum dilutions in the range 1:1 to 1:1600 with no suggestion that dilutions outside these limits would result in failure. Typically the fluid is incubated with the immobilised antigen for up to 30 minutes at 37°C but times of 15 minutes may be used. After step (b) excess fluid is removed by known methods, eg: washing with PBS.

Step (c) of the first option is typically carried out by incubating the binding reagent, eg: antigen, with a solution containing gold labelled secondary binding reagent, eg: secondary antibody, eg: containing 5% AUROPROBE, gelatin (eg: 5% to minimise non-specific binding) and 0.1% goat serum in PBS, at 37°C for 15 minutes. This protocol is an optimum for use with a substrate in the form of microtitre cells containing a coating of 300ng of IgG. The incubation period does not appear to be



critical between 5 and 30 minutes at high anti-HIV titres but low HIV positive sera need about 10 minutes incubation to achieve maximum binding. Fig 2 of the accompanying drawings shows the effect of different AUROPROBE concentrations and incubations with respect to the final absorbance of the wells after silver enhancement. After step (c) excess gold labelled antibody solution is removed by known methods, eg: washing with demineralised water.

The silver ion exposure steps (d) and (e) are typically carried out by exposing the immobilised specific binding agent, eg: antigen, to a solution of silver ions, eg: IntenSE\*II made up to suppliers specifications at room temperature for 15 minutes, preferably with shaking. The time required to reach maximum absorbance in an anti-HIV assay is found to be dependent on the anti-HIV titre. During the first 20 minutes or so the silver precipitation process is specific for gold particles, thereafter self nucleation begins to occur.

As mentioned above the presence of the target analyte in the first option is manifested by the appearance of a visible brown or a black colouration, the intensity of which may be quantitatively measured by absorbance measurement or comparison with standard colour charts. It is preferred to carry out multiple assays and to compare these with standards and controls.

The specific volumes, etc, used in the assay method of the invention will of course vary with the particular circumstances, eg: the nature of the substrate, but is adaptable to work on a microlitre scale using microlitre well substrates.

The kits for performance of the method of the invention may be



provided in a number of forms and ways of adapting immunoassay methods into kit form are well known. One form of kit may comprise a device similar to the known HIVCHEK device, in which the substrate is a porous membrane on which is immobilised an antigen which includes a recombinant fragment of an HIV protein, backed by an absorbent pad to encourage capillary transport of liquids applied to the opposite side of the membrane.

Typically for a kit in this form of the first option, a buffer (supplied as part of the kit) is first applied to the membrane followed by a fluid sample and then a further application of buffer. The membrane is treated with a wash solution followed by an application of a solution containing gold labelled antibody, both being part of the kit, and a further wash step. Finally silver ion solution is applied from the kit before observation of the appearance of any darkening of the membrane. The various applications of these solutions may be interspersed with appropriate incubation periods.

The invention will now be illustrated by way of example only with reference to the Figure 1, which shows the chemical basis of the method as applied to HIV in the first non-competitive option ; Fig 2, which shows graphically the effect of increasing AUROPROBE percentages and step (c) incubation times; Fig 3, which shows test results and Fig 4, which shows the effect of dilution of the serum sample; Figs 5 and 6, which show the absorbance at 450nm of assay microtitre wells of ELISA and SEGLISA assays carried out upon the same HIV whole blood samples; Figs 7 and 8, which show histograms of 50 positive and 50 negative HIV sample results from a SEGLISA; Figs 9 and 10, which show compared

results from assays run with whole blood and serum samples for HIV antibody assay; Fig 11, which shows a histogram for the effect of incubation time of the sample in the microtitre wells with serum and whole blood; Fig 12, which shows a schematic diagram of the binding basis of a SEGLISA assay for theophylline; Fig 13, which shows the binding basis for a SEGLISA assay for Rubella antibodies; Figs 14 to 21, which show the effect of gold conjugate dilution, gold particle size, incubation time with gold conjugate, gold conjugate solution volume, amount of gelatin in the conjugate solution, type of silver enhancer kit used, incubation time with silver enhancer and volume of silver enhancer on the assay result as measured by microwell absorbance.

Referring to Fig 1, the principle of the enhancement method is shown with regard to the first option. An antigen (1) is immobilised onto a solid polystyrene substrate (2). A human anti-HIV antibody (3) has been captured by the antigen (1) and is therefore itself immobilised on the substrate (2).

The antibody (3) has been exposed to an anti-human IgG (4) labelled with a colloidal gold particle (5) and thus labelled IgG (4) has become bonded to the antibody (3). The antigen-antibody-IgG (1)(3)(4) complex has been exposed to a solution containing silver ions and these have become nucleated around the gold particles (5) as solid silver particles (6) having a dark colour to the eye. The term SEGLISA is used herein to indicate use of the present Silver Enhanced Gold Labelled Immunosorbant Assay.

The method of the invention will now be further illustrated by way of the Examples from which it will be clear to a man skilled

in the art that many other possibilities may be derived for assay of other target analytes within the scope of the invention.

EXAMPLE 1. SEGLISA ASSAY OF HIV IN HUMAN BLOOD SERUM.

**Materials:** Except where otherwise stated laboratory reagents were obtained from BDH Ltd, Poole, Dorset, BH12 4NN. Antigen-coated microwell strips were a gift from DuPont (UK) Ltd, Stevenage, UK. and are usually sold as part of the DuPont HTLV-III/LAV ELISA kit.

The gold and silver reagents were supplied by Janssen Life Sciences, Wantage, Oxon, OX12 0DQ. These consisted of goat anti-human IgG labelled with colloidal gold particles of 10nm mean diameter (AUROPROBE BL plus GAHu) and IntenSE\*II (product No 30.653.01), a silver enhancement reagent kit that works well under ambient light conditions. Both reagents still worked well after one year's storage; the AUROPROBE at 4°C and the IntenSE\*II at room temperature. AUROPROBE and IntenSE\*II are Janssen registered trade marks.

**Specimens:** In order to assess the new assay a panel of samples consisting of known anti-HIV positives and negatives were compiled. The negative sera were obtained from specimens sent for tests other than HIV testing. The positives were from patients being investigated or treated for HIV infection, persistent generalised lymphadenopathy, AIDS related complex or AIDS. As a preliminary to this study all the samples in the panel were tested and categorised as being either positive or negative. this involved screening by a commercial ELISA kit and confirmation of positive results by the Virus Reference

Laboratory (Colindale Avenue, London) using complementary tests. The Central Public Health Laboratory Division of Microbiological Reagents and Quality Control (DMRQC) supplied six plasma controls (panel No 8) consisting of five positives, prepared by diluting a high titre positive specimen, and one negative. Another DMRQC control preparation (designated LP3), which was a positive sample diluted to the point where it gave neither a strong positive or negative reading, was used as a reference serum.

**Apparatus:** Standard laboratory ELISA equipment was used. The absorbance of the microtitre wells was read using a Dynatech MR250 (registered trade mark) plate reader fitted with a 450nm filter. A special grade 2 microbiological safety hood was used.

**Protocol:** One hundred microlitres of phosphate buffer saline (PBS) (0.14 M NaCl, 2.7 mM KCl, 1.5 mM  $\text{KH}_2\text{PO}_4$ , 8.1 mM  $\text{Na}_2\text{PO}_4$ , pH 7.3) containing 10% of normal goat serum and 0.5% Tween 20 (registered trade mark) was added to each well of a standard ELISA microtitre well plate and the plates left to stand for a minimum of 2 minutes at room temperature. 50 microlitres of the PBS was then removed from each well and 50 microlitres of test serum (or where appropriate reference serum or PBS) was then added. Each strip would typically contain one reference and one blank well. The wells were sealed, agitated for 10 seconds and then incubated at 37°C for 15 minutes before being washed five times with 300 microlitres of PBS containing 0.1% goat serum. 50 microlitres of the gold conjugate reagent was then added (this solution contains 5% AUROPROBE, 5% gelatin and 0.1% goat serum in PBS). The wells were sealed and agitated as before and then incubated at 37°C for 15 minutes. After washing again as above

with demineralised water (3 x 300 microlitres), 200 microlitres of a freshly made up solution of silver enhancer solution (made up by mixing equal volumes of the two solutions comprising the IntenSE\*II kit) was added to the wells and the wells were shaken at room temperature for 15 minutes. The absorbance of the wells was then measured and known negative and positive controls were included in each batch.

HIV positive samples produced a thin black high-contrast coating on the well bottom and sides (absorbance typically greater than 1) while very dilute positive sera gave a brown discolouration to the wells. With negative sera there was no visible staining.

Development of silver enhancement: Experiments to discover the effects of variables on the time-consuming incubation stages were carried out with the aim of designing a batch assay capable of producing results in a relatively short time. Some observations are summarised below.

Antigen/anti-HIV reaction: Neat test serum was diluted by adding it to microwells containing an equal volume of buffer. Goat serum in the buffer was necessary to prevent non-specific binding, however, it also decreased the rate of reaction. It was found that by adding the buffer containing the goat serum before the test serum (at least 2 minutes) that non-specific binding was prevented using a lower concentration of said goat serum than would otherwise be the case.

Decreasing the incubation time from 30 minutes to 15 minutes produced no decrease in absorbance thus it may be possible to decrease the reaction time further but the need for precise

timing between manually loading each well and reading it makes very short incubation periods more difficult to cope with.

Results: A panel of 61 samples comprising 30 HIV negative and 31 HIV positive samples was tested by SEGLISA. The DMRQC control designated LP3 was used as a reference (cut-off standard).

The results are presented in terms of the ratio of sample absorbance/reference absorbance. This S/R ratio is equal to 1 when the absorbance of test sample equals that produced by the reference serum. The test ratios are illustrated graphically in Fig 3.

Positive and negative sera fell into two clearly discernable groups. For the negative sera the mean S/R was 0.31 while for the positives this was 2.72. Mean positives/mean negatives gave a value of 8.66. The DMRQC controls were also correctly categorised (ie: one negative and five positives).

As with all anti-HIV test procedures the sensitivity and specificity of the assay is influenced by the chosen levels of the cut-off values; unfortunately there are no absolute standards. As the threshold for defining the minimum positive SEGLISA ratio decreases the sensitivity of the assay in detecting weak anti-HIV cases increases while the specificity decreases. Since the positive results found by a screening test would normally be confirmed by several other tests of different formats it is best to bias the cut-off towards producing false positives rather than false negatives. For example, for the present assay we would suggest a cut-off ratio of 0.8 for use in blood screening. At this ratio the cut-off should not unnecessarily exclude large quantities of donated blood.



If we define sensitivity of the assay as the proportion of the positive sera correctly identified then the test has a sensitivity of 100%; if the assay specifically is defined as the proportion of negatives correctly identified then the specificity was 100%.

**Analytical sensitivity:** The ability of the assay to detect low concentrations of HIV antibodies was assessed by titrating two anti-HIV positive sera. The positive sera were diluted in negative serum to give dilutions of 1:100, 1:200, 1:400, 1:800 and 1:1600. At dilutions of 1:1600 both sera still gave positive results. As can be seen from Fig 4 the slope of the curve is steepest at high sample dilutions. This gives maximum sensitivity to the detection of sera with very low antibody titres.

#### EXAMPLE 2. SEGLISA ASSAY OF HIV IN WHOLE HUMAN BLOOD.

**Methods and materials:** HIV antigen coated wells and positive and negative HIV antibody controls were supplied by Mercia diagnostics Ltd, Anti-HuIgG conjugate (AUROPROBE LM GaHu) was supplied by Janssen Life Sciences, Anti-HuIgG-Horse radish peroxidase and silver enhancing solution (SE-100) and all other reagents were supplied by Sigma Chemical Company. Storage of reagents was at 4°C except for raw chemicals for silver enhancement solutions which were stored at room temperature in the dark. All experiments were carried out in duplicate with results quoted as the mean absorbances between the two. The cut-off value for a positive and a negative HIV antibody level was taken as the mean absorbance of the positive HIV antibody



control divided by two. Absorbances of tests above this value were termed positive for HIV antibodies and below this value as negative for HIV antibodies.

**Specimens:** A panel of 50 known anti-HIV positive samples were collected from blood specimens sent into the laboratory from patients suffering from HIV infection. 50 blood samples were also collected from specimens sent into the laboratory for other biochemical analysis.

**Apparatus:** Standard laboratory micro-titre equipment was used. All testing was carried out under a special grade 2 micro-biological safety hood. The absorbance of the micro-titre wells was read using a Dynatech MR250 plate reader fitted with a 450nm filter.

**Assay procedure:** Test sera and controls were diluted 1 in 20 and test whole blood 1 in 10 with specimen diluent (8g NaCl, 0.2g  $\text{KH}_2\text{PO}_4$ , 2.9g  $\text{NaH}_2\text{PO}_4 \cdot 12\text{H}_2\text{O}$  + 1% BSA + 0.05% Tween 20 made up to 1 litre using distilled water), 200 microlitre of each was added to micro-titre wells pre-coated with inactivated HIV antigens, the wells were incubated at 37°C for 60 minutes. The serum or whole blood was then aspirated off and the wells were washed 5 times in wash buffer (8g NaCl, 0.2g  $\text{KH}_2\text{PO}_4$ , 2.9g  $\text{NaH}_2\text{PO}_4 \cdot 12\text{H}_2\text{O}$  + 0.05% Tween 20 made up to 1 litre using distilled water). The antibody /antigen complex could then be quantified by either ELISA or SEGLISA.

**SEGLISA:** 100 microlitres of IgG-Gold conjugate (diluted 1 in 50 with specimen diluent) was added to each of the wells, incubation was again at 37°C for 60 minutes. The wells were then washed 3 times with wash buffer and silver enhanced (100

microlitres of equal volumes of initiator A and enhancer B) for 30 minutes at room temperature. After washing 3 times in distilled water the absorbances of the wells were read at 450nm.

**Comparative ELISA:** 100 microlitres of Anti-HuIgG-Horse radish peroxidase conjugate (diluted 1 in 200 with specimen diluent) was added to each well and again incubated at 37°C for 60 minutes. The conjugate was aspirated off and the wells were washed 3 times in wash buffer. The substrate was made up 10 minutes prior to use by diluting Tetramethylbenzadine chromogen (TMB) 1 in 100 with substrate solution (citrate buffer + H<sub>2</sub>O<sub>2</sub>). This was incubated on the wells at room temperature for 30 minutes after which time 25 microlitres of 2 M H<sub>2</sub>SO<sub>4</sub> was added to stop the reaction. The wells were then read within 30 minutes for absorbance at 450nm.

**Results:** 15 blood samples shown to be negative for HIV antibodies were tested using serum and whole blood using the ELISA and SEGLISA methods descibed above. Table 1 shows a summary of the results obtained.

TABLE 1.

<u>Assay</u>	<u>Serum</u>		<u>Whole blood</u>	
	+	-	+	-
Cut-off Abs.	0.213	0.210	0.388	0.378
ELISA	0	15	12	3
SEGLISA	0	15	0	15

+ = a positive result; - = a negative result. Cut-off value for positive and negative results is the mean absorbance of the positive control divided by two. Readings were at 450nm.

Figures 5:ELISA and 6:SEGLISA show this result in more detail. The ELISA correctly assigned all 15 samples negative when using the serum, however when using whole blood 12 samples were given as falsely positive, with all 15 showing absorbances at least double those obtained from equivalent serum samples. The SEGLISA correctly identified all 15 samples as being negative for HIV antibodies when using both serum and whole blood methods. These results show that a HRP-ELISA is unsuitable to test whole blood specimens, whereas SEGLISA reliable results unaffected by the presence of the red cells.

50 known positive HIV Ab and 50 known negative HIV Ab whole blood samples were tested by a SEGLISA. Table 2 shows the results obtained.

TABLE 2.

	Positive	Negative
Positive batch		
No. of samples	50	0
Negative batch		
No. of samples	0	50

Cut-off value for positives was 0.362 and for negatives was 0.368. Figures 7 and 8 show histograms of the negative and positive screen respectively. All 50 negative patient samples give an absorbance of below 0.362 and all 50 positive samples give an absorbance above the cut-off value of 0.368.

**Precision:** A positive HIV Ab whole blood and a serum sample were assayed by a SEGLISA 16 times. All aspects of the assay were kept constant. The results from these precision tests are shown in Table 3.

TABLE 3.

	Serum	Whole blood
Number of entries	16	16
Mean	0.502	0.539
SD	0.044	0.041
C of V	8.7%	7.5%

The mean given is an average absorbance of all the individual results, SD is the standard deviation of the set of results and C of V is the correlation of coefficient. The whole blood SEGLISA gives a very respectable C of V of 7.5% which indicates a good precision. It is also comparable to the serum SEGLISA which gives a C of V of 8.7%, suggesting that the whole blood does not have a varied effect on the assay.

**Sensitivity:** The sensitivity of the assay was measured by using various titrations of a positive and negative anti-HIV whole blood sample and comparing the results with the equivalent serum test. Serum was diluted 1:10, 20, 40, 80, 160, 320, 640, 1280, 2560, 5120, 10240, 20480 and 40960 with specimen diluent. the blood cell fraction of a negative HIV Ab sample was used to add to each dilution to keep the serum/blood cells ratio at 1:1 whilst maintaining the quoted dilutions, with PBS buffer added for the equivalent serum assay.

Figure 9 shows the results obtained from the whole blood assay, on dilution of the positive sample we see that it becomes negative at 5120 dilution factor. These results are comparative with the serum assay, as shown in Figure 10, which becomes negative at a serum dilution factor of just above 5120. These

results show that sensitivity is not lost when using whole blood.

Incubation times of whole blood: A positive and a negative HIV Ab sample was tested as whole blood and as serum with varying incubation times: 0, 5, 10, 25, 30, 45, 60 and 120 minutes were tested. The whole blood was also observed to record it's appearance in the wells after each incubation time as shown in Table 4.

TABLE 4.

Minutes	State
5	Totally mixed.
10	Totally mixed.
15	Totally mixed.
30	Slight serum layer at top fifth of blood.
45	Diffuse serum layer at top third of blood.
60	Diffuse serum layer at top half of blood.
120	Layer of cells at bottom fifth of blood.

Figure 11 shows a histogram of the results obtained. An increase of absorbance is seen as incubation times increase, with serum giving slightly higher absorbances than whole blood. It is therefore shown that the separating affect of cells and serum/buffer during incubation on wells, has no significant effect on the immunological reaction.

EXAMPLE 3. SEGLISA ASSAY OF HIV IN WHOLE BLOOD SAMPLES  
BLOTTED ONTO FILTER PAPER.

A test was developed to see if antibodies could be extracted

from whole blood that had been spotted onto an absorbant carrier material, eg: filter paper, with the aim of providing a 'far station' test submission procedure whereby patients could submit samples by post; as there is no requirement for the analyte being determined to be a viable part of the infection any characteristic compound may be assayed.

Guthrie-PKU test filter paper was used as the spotting medium. 1 cm<sup>2</sup> squares were cut out and placed at the bottom of 5 ml tubes. 0.1 micrograms/ml solution of IgG labelled with I<sup>125</sup> was diluted 1:10 using whole blood. 20 microlitres was then added to each of 5 tubes leaving one blank. All 6 tubes were immediately counted for gamma emission on a NE 1600 gamma counter for 120 seconds. The tubes were then placed into a 37°C incubator for 60 minutes to dry. After this time 500 microlitres of PBS-BSA-Tween was added to each tube and vortex mixed for 1 minute before being agitated for 15 minutes. The buffer was then aspirated off and placed in a separate tube, both filter paper and buffer were then counted for gamma emission for 120 seconds. Results are displayed in Table 5.

TABLE 5.

Tube	Blank	1	2	3	4	5	Average
Totals	98	1282	1229	1239	1269	1248	1248
Filter	103	261	241	218	247	207	235
Buffer	143	960	992	1096	1089	1021	1032

Percentage bound is 100%

After extraction percentage left on the filter paper = 235/1248

x 100 = 18%

After extraction percentage left in the buffer =  $1032/1248 \times 100$   
 = 82%

A G-75 Sephadex column was used to separate out the IgG tracer used in the experiments and it was found that 78% of the tracer was bound to the antibody. 80% of the counts had been extracted from the filter paper thus the proportion of the antibody extracted is found by  $58/(58+20) \times 100 = 74\%$ . Having shown the viability of extracting the antibody a SEGLISA was carried out as follows:

Guthrie-PKU test filter paper was used as a spotting medium whereby 1 cm<sup>2</sup> squares were cut out and placed at the bottom of 5 ml tubes and whole blood samples positive for HIV antibodies and 15 whole blood samples negative for HIV antibodies were tested against controls of positive and negative HIV antibody serum. 20 microlitres of each sample was then spotted onto the filter papers in the tubes. All the tubes were then sealed and allowed to stand overnight to dry. After this time 400 microlitres of PBS-BSA-Tween was added to each tube and mixed on a sero-agitator for 30 minutes. 200 microlitres of the buffer was then aspirated off and placed in a HIV antigen coated well in order to carry out a SEGLISA by the method of Example 2. with results as set out in Table 6.

TABLE 6.

Specimen	1	2	3	4	5	6	7
Positive Abs <sub>450</sub>	0.610	0.721	0.511	0.492	0.811	0.510	0.595
Negative Abs <sub>450</sub>	0.127	0.134	0.114	0.115	0.125	0.099	0.142



TABLE 6 (cont.)

Specimen	8	9	10	11	12	13	14
Positive Abs <sub>450</sub>	0.611	0.734	0.131	0.710	0.739	0.512	0.687
Negative Abs <sub>450</sub>	0.090	0.152	0.131	0.115	0.110	0.106	0.113
Specimen	15	Control					
Positive Abs <sub>450</sub>	0.743	0.788					
Negative Abs <sub>450</sub>	0.121	0.138					

All results have been corrected by a background factor of 0.109 derived from the present and previous means of the negative results.

EXAMPLE 4. A COMPETITIVE SEGLISA FOR THEOPHYLLINE (A DRUG) IN SERUM.

An IDS theophylline ELISA was used in part for the SEGLISA and in full for a comparative ELISA. Anti-mouse IgG-Gold (BL grade) was supplied by Janssen Life Sciences Ltd while silver enhancement was carried out using a Sigma SE-100 supplemented with 0.5% hydroquinone and 0.1% gum acasia (designated SigNish). Standards, controls and patient sera were diluted 1:30 with assay buffer (PBS-1%BSA-0.5%Tween). 50 microlitres of diluted sample was then added to the microtitre wells precoated with BSA-theophylline (from the IDS kit). 200 microlitres of anti-theophylline monoclonal antibody (diluted 1:500 with assay buffer; also IDS) was then also added to each well. The wells were incubated for 30 minutes at room temperature and further washed 3 times in wash buffer (PBS-0.5% Tween). Any antibody could then be quantified either using the ELISA or the SEGLISA.

Comparative ELISA: 200 microlitres of anti-mouse IgG-Horse

radish peroxidase (diluted 1:200 in assay buffer) was added to each well and incubated for 30 minutes at room temperature. After 3 washes with wash buffer, 200 microlitres of substrate solution was incubated for 10 minutes at room temperature (made up immediately prior to use by adding equal volumes of  $H_2O_2$  solution and TMB solution). The reaction was stopped by adding 100 microlitres of 2M  $H_3PO_4$  and the absorbance read at 450nm.

SEGLISA: 50 microlitres of anti-mouse IgG-Gold (diluted 1:25 in assay buffer) was added to each well and incubated for 30 minutes at room temperature. After 3 washes with wash buffer the wells were silver enhanced using 100 microlitres of a SigNish comprising equal volumes of initiator A (SE-100 kit) with 0.1% gum acacia, 0.5% hydroquinone and enhancer B. After 20 minutes at room temperature the wells were washed 3 times with deionized  $H_2O$ , blotted dry and their absorbances read at 450nm. A schematic diagram of the mechanism of this assay is shown in Figure 12.

A 5 microgram/ml sample of theophylline was tested on the competitive SEGLISA and repeated up to 16 times with all the parameters being kept constant between each test. 50 microlitres of the sample (x30 dilution) was added to 16 BSA-theophylline coated wells and agitated at room temperature for 60 minutes. IGSS was with 50 microlitres of anti-IgG-Gold x 20 dilution added to each well at room temperature for 60 minutes and followed by 20 minutes of 100 microlitres of SigNish before reading at 450nm. Results are displayed in Table 7 and give reproducibility of this SEGLISA. A schematic diagram of the assay is shown in Figure 12 wherein (1) is bound theophylline, (2) is sample theophylline, (3) is anti-theophylline monoclonal antibody, (4) is antimouse

IgG-gold, (5) the gold in that, (6) silver deposit and (7) wells.

TABLE 7.

Sample	1	2	3	4	5	6	7
Abs <sub>450</sub>	0.513	0.426	0.466	0.432	0.507	0.516	0.428
Sample	8	9	10	11	12	13	14
Abs <sub>450</sub>	0.480	0.472	0.486	0.422	0.425	0.437	0.487
Sample	15	16					
Abs <sub>450</sub>	0.433	0.421					

Mean optical density was 0.459 with a standard deviation of 0.035 and a Coefficient of Variance of 7.7%. Further experiments demonstrate that 100 microgram/ml samples of theophylline diluted by 30 times in assay buffer and assayed as above gave a mean optical density for 16 tests of 0.045 whereas similarly diluted 0.1 microgram/ml samples gave a mean of 1.007 thus demonstrating the sensitivity of this competitive SEGLISA.

The SEGLISA above was further applied to samples of theophylline made up in assay buffer, human serum, human plasma and serum and the results compared as shown in Table 8.

TABLE 8.

Conc microlitres/ml	0	2.5	5	10	25	33	100
Assay buffer sample							
Abs <sub>450</sub>	0.817	0.763	0.697	0.598	0.200	0.180	0.051
Human serum sample							
Abs <sub>450</sub>	0.859	0.824	0.783	0.643	0.213	0.100	0.102
Human plasma sample							
Abs <sub>450</sub>	0.869	0.563	0.376	0.226	0.091	0.089	0.073
Horse serum sample							
Abs <sub>450</sub>	0.884	0.798	0.643	0.466	0.159	0.060	0.043

The SEGLISA assay of this example was finally used in comparison with a standard HPLC measurement of ten samples of serum from patients under aminophylline treatment. Silver enhancement time was increased in this case to 40 minutes. Results are shown in Table 9 and relate to values of theophylline micrograms/litre.

TABLE 9.

Patient Sample	1	2	3	4	5	6	7	8	9	10
SEGLISA	15.5	17.5	15.0	18.0	17.5	19.5	5.4	35.6	8.7	22.5
HPLC	14.1	16.0	14.7	14.9	16.5	16.2	5.2	33.9	7.3	20.8

EXAMPLE 5. SEGLISA ASSAY OF HUMAN GROWTH HORMONE IN SERUM.

In order to demonstrate the ability to determine levels of hormone target analytes by the method of the invention a sandwich type SEGLISA was carried out for human growth hormone (hGH) as follows and compared to an ELISA for said hormone.

Procedure: 50 microlitre standards (Horse serum + 0.05% Sodium azide), controls and patient samples were added to IDS microtitre wells precoated with antibodies to human growth hormone. 150 microlitres of hGH monoclonal antibody was also added to each well (diluted 1:10000 in PBS-1%BSA-0.5%Tween). The wells were then incubated for 120 minutes at room temperature. After washing three times with wash buffer (PBS-0.5% Tween) 100 microlitres of anti-mouse IgG-Gold (Amersham UK Ltd. BL grade, diluted 1:25 in PBS-1%BSA-0.5%Tween) was added to each well and incubated at room temperature for 60 minutes. After a further three washes the wells were silver enhanced with 100 microlitres

of SigNish (comprising SE-100 + 0.5% Hydroquinone + 0.5% gum acasia) for 20 minutes at room temperature, washed with distilled water and read for their absorbance at 450nm. Precoated wells and monoclonal antibodies were supplied by Immunodiagnostics Systems (UK) (IDS).

Comparative ELISA: 50 microlitres of standards (Horse serum), controls and patient samples were added to hGH antibody coated wells. 150 microlitres of hGH monoclonal-Horse radish peroxidase conjugate was then added to each well (1:10000 in PBS-1%BSA-0.5% Tween). Incubation was for 120 minutes at room temperature. After washing three times with wash buffer 300 microlitres of colour reagent was added to each well (equal volumes of reagents A and B from the ELISA kit prior to use) and incubated at room temperature for 10 minutes. 100 microlitres of stop reagent was then added to each well and their absorbances read at 450nm.

A hGH SEGLISA and ELISA were carried out on 10 patient samples and the results were then read from a standard curve and correlations carried out between the two. The results are shown in Table 10.

TABLE 10.

Conc mIU/litre of hGH	1	2	3	4	5	6	7	8	9	10
ELISA	26.3	0.40	35.3	11.2	0.30	2.00	17.1	0.3	16.5	18.0
SEGLISA	25.2	0.40	33.5	10.0	0.40	1.20	16.6	0.5	15.7	17.2

Correlation coefficient 0.9996

Figure 13 is a schematic diagram of the mechanism of this assay wherein (1) is bound anti-hGH, (2) is hGH, (3) is

monoclonal antibody to hGH, (4) is anti-IgG-Gold conjugate, (5) is the gold label in that conjugate, (6) is silver deposited onto said gold and (7) is the well surface.

EXAMPLE 6. SEGLISA ASSAY FOR RUBELLA ANTIBODIES IN HUMAN SERUM.

In order to demonstrate the ability to determine levels of antibodies and thus the presence of infections other than HIV by the method of the invention a SEGLISA was carried out for Rubella antibodies. Rubella is sometimes very hard to diagnose due to failure to exhibit symptoms and, due to it's seriousness if contracted by mothers during pregnancy, assays capable of detecting it are of great assistance.

**Methods and materials:** A standard ELISA kit (J Seacombe, Mercia diagnostics, Captia Rubella-G) was used for comparative ELISA tests and components of it were used for the SEGLISA. Anti HuIgG-Gold conjugate was supplied by Janssen and was used with 5nm gold particle size. Silver enhancer solutions were supplied by Janssen and Sigma Chemical Co. All other chemicals and reagents were supplied by Sigma unless stated. Storage of reagents was at 4°C for all except the raw chemicals for the silver enhancement solution which were stored in the dark at room temperature. Amount of rubella antibody is given by it's antibody index which is the ratio of the OD test/OD cut-off value of the kit. The cut-off value is derived from clinical trials as the value giving discrimination between positive and negative sera.

**Assay procedure for rubella antibody detection:** Test sera and kit controls were diluted 1 in 100 with specimen diluent

(PBS-1%BSA-0.05%Tween), 100 microlitres of each was added to microtitre wells precoated with inactivated rubella antigens (Mercia Diagnostics), the wells were incubated at 37°C for 60 minutes and then the serum was aspirated off and the wells washed three times with wash buffer (1 in 20 dilution of the kit wash buffer- PBS-0.05%Tween).

SEGLISA: 100 microlitres of IgG-Gold conjugate (diluted 1 in 50 with specimen diluent) was then added to each of the wells, incubation was again at 37°C for 60 minutes and then the wells were washed three times with wash buffer and silver enhanced (100 microlitres of equal volumes of kit initiator A (supplemented with 0.05% gum acasia and 0.5% hydroquinone) and enhancer B) for 20 minutes at room temperatures. After washing three times in distilled water the absorbances of the wells were read at 450nm.

ELISA: 100 microlitres of AntiHuIgG-Horse radish peroxidase conjugate (diluted 1 in 200 with specimen diluent) was added to each well and again incubated at 37°C for 60 minutes. The conjugate was aspirated off and the wells were washed three times with wash buffer. The substrate was made up 10 minutes prior to use by diluting Tetramethylbenzadine chromogen (TMB) 1 in 100 with substrate solution (citrate buffer + H<sub>2</sub>O<sub>2</sub>). This was incubated at room temperature for 30 minutes after which time 25 microlitres of 2M H<sub>2</sub>SO<sub>4</sub> was added to stop the reaction. The wells were then read within 30 minutes for absorbance at 450nm. The results of the two assays on 84 serum samples are provided in Table 11 wherein the AI value is derived from the OD<sub>450</sub> of the sample/ OD<sub>450</sub> of the Low positive titre (cut-off value).



TABLE 11.

Sample No	ELISA AI	SEGLISA AI	Sample No	ELISA AI	SEGLISA AI
1	3.61	3.65	46	3.04	3.10
2	2.94	2.88	47	0.33	0.52
3	3.56	2.93	48	4.75	3.46
4	4.34	3.56	49	3.58	3.53
5	4.17	3.47	50	3.19	2.98
6	0.44	0.82	51	5.07	3.22
7	3.20	2.87	52	4.20	3.44
8	5.41	3.74	53	2.84	2.85
9	3.62	3.11	54	3.09	2.51
10	0.28	0.62	55	3.58	2.76
11	2.68	2.86	56	2.48	2.51
12	5.07	3.74	57	0.29	0.49
13	3.76	2.94	58	3.93	2.28
14	4.24	3.20	59	2.96	2.23
15	0.29	0.85	60	3.56	3.75
16	0.24	0.89	61	0.32	0.74
17	2.60	2.69	62	4.27	2.90
18	1.76	2.15	63	1.80	2.10
19	3.68	2.69	64	4.19	3.25
20	5.28	2.15	65	2.29	2.03
21	5.24	2.69	66	5.12	3.06
22	3.56	3.14	67	5.79	2.98
23	4.13	3.38	68	0.28	0.47
24	4.72	3.29	69	5.10	2.93
25	1.60	2.59	70	0.99	1.95
26	2.64	2.42	71	4.34	2.94
27	3.82	2.07	72	0.29	0.82
28	4.68	2.44	73	3.26	2.71
29	1.90	2.98	74	3.71	2.90
30	5.35	2.66	75	4.68	3.39
31	2.80	2.24	76	4.43	2.93
32	0.48	3.21	77	5.62	3.23
33	4.16	2.34	78	4.84	3.61
34	3.42	0.76	79	0.34	0.79
35	2.44	2.46	80	4.95	2.60
36	1.81	3.03	81	4.52	2.96
37	3.85	2.00	82	2.61	2.46
38	5.50	1.78	83	3.84	2.82
39	4.04	3.37	84	3.54	2.20
40	2.63	3.54			
41	3.95	3.39			
42	0.80	2.18			
43	5.10	3.26			
44	3.40	1.98			
45	1.95	3.48			

TABLE 11 (cont.)

Total positives ELISA: 71 SEGLISA:73  
Total negatives ELISA: 12 SEGLISA:11  
Total equivocal ELISA: 1 SEGLISA: 0

EXAMPLE 7. OPTIMISATION OF PARAMETERS FOR SEGLISA.

In order determine optimal parameters for the SEGLISA assay standard experiments were carried out using microtitre plates coated with Human ImmunoglobulinG (HuIgG) to bind antibody-gold-conjugate specific for it and then enhancing this using a variety of enhancing conditions.

Methods and materials: Microtitre plates were supplied by Greiner Co. Protein A-Gold conjugate and SE-100 silver enhancement kit was supplied by Sigma Chemical Co in 5, 10 and 20 nm gold particle sizes. Anti-HuIgG-Gold conjugate (AUROPROBE GAHu G5) and IntenSE M and IntenSE BL silver enhancement kits were supplied by Janssen Life Science Products Ltd. All other chemicals unless stated were supplied by Sigma Chemical Co. Storage of chemicals and reagents was at 4°C for all except the neat Protein A-Gold conjugate which was stored at -20°C. All tests were carried out in duplicate and average absorbances were used.

Coating of microtitre plates with HuIgG: A 96 well microtitre plate was coated with purified Human Immunoglobulin G (HuIgG). 1mg of HuIgG was dissolved in 1 ml of coating buffer (1.59g Na<sub>2</sub>CO<sub>3</sub>, 2.93g NaHCO<sub>3</sub>, 0.2g NaN<sub>3</sub> made up to 1 litre with demineralised water and adjusted to pH 9.6). The 1 mg/ml stock solution was diluted further to give the desired HuIgG concentrations. The absorbance of the HuIgG solution was read at

280nm. 200 microlitres of the solution was added to each well and the plate was left overnight to incubate at 4°C. After incubation the absorbance of the HuIgG solution from the wells was read again at 280nm. Calculations of the amount of Human IgG absorbed to the plate gave a range of between 200 and 300ng HuIgG per well. The plate was washed with PBS + 1%BSA(8g NaCl, 0.2g KH<sub>2</sub>PO<sub>4</sub>, 2.9g NaH<sub>2</sub>PO<sub>4</sub>.12H<sub>2</sub>O + BSA made up to 1 litre using demineralised water) three times then left for one hour with 250 microlitres PBS + 1%BSA per well at room temperature. After this time the plate was washed again three times with PBS + 1% BSA + 0.5%Tween 20 (8g NaCl, 0.2g KH<sub>2</sub>PO<sub>4</sub>, 2.9g NaH<sub>2</sub>PO<sub>4</sub>.12H<sub>2</sub>O + 1% BSA + 0.5%Tween made up to 1 litre using demineralised water) and allowed to dry. The plate was then immediately stored at 4°C.

SEGLISA: 50 microlitres of protein-A conjugate diluted with PBS + 1% BSA + 0.5% Tween was added to each well and incubated at room temperature for 20 minutes. The plate was then washed three times with PBS + 1% BSA + 0.5% Tween 20 and then allowed to dry. 200 microlitres of freshly made up silver enhancer solution (equal volumes of Enhancer A and Initiator B) was added to each well and the wells left for 15 minutes. After this time the enhancer was washed off with demineralised water and the wells were immediately read on a plate reader at 520nm.

RESULTS: In order to optimise the SEGLISA various parameters were studied using constant values for parameters not under study. The results of the studies are summarised in the Figures 4 through 23. These results provide the following findings:

Figure 14: Shows Absorbance values for a SEGLISA performed as above using various dilutions of gold conjugate from 1:10 to 1:200 in PBS + 1%BSA + 0.5% Tween 20. The plot shows a plateau between dilution factors of 10 to 50 whereby it can be seen that a dilution factor of 50 is the most efficient.

Figure 15: Shows Absorbance values using various particle sizes for the gold in the conjugate whereby it is seen that 5nm gold particles give the highest absorbance whereas 20nm gold particles give virtually no absorbance.

Figure 16: Shows absorbance values using various incubation times with the gold conjugates from 0 to 60 minutes: 30 minutes proving to be the optimum time.

Figure 17: Shows absorbance values using various volumes of gold conjugate solution from 0 to 100 microlitres. 5, 10 and 20 microlitres volumes were not sufficient to cover the whole of the bottom of the flat microtitre well; 50 and 100 microlitre volumes give high absorbances with the wells being fully covered resulting in an even conjugate and thus even silver deposit.

Figure 18: Shows the effect of varying the amount of gelatin in the conjugate solution (as a colloid stabiliser); no significant difference was found for gelatine additions of 0 to 0.5%.

Figure 19: Shows the absorbance obtained using equivalent samples with SEGLISAs using three different silver enhancement kits; IntenSE M gave the highest absorbance with a mirrored black deposit. The Sigma kit did give a mirrored black deposit of satisfactory nature when stabilised with hydroquinone and gum acasia as used in the other Examples herein.

Figure 20: Shows the effect of incubation time with the silver enhancer for each of the kits; 20 to 30 minutes provides a high enough absorbance to work with before self nucleation sets in.

Figure 21: Shows a steady increase in absorbance as volume of enhancer is increased up to 100 microlitres.

EXAMPLE 8. SUITABLE LABORATORY PREPARED SILVER ENHANCER REAGENT.

In order to provide a non-commercially available, laboratory preparable alternative silver enhancer solution experiments were carried out on the HIV immunogold procedure and the following reagent kit was found to be suitable comprising four reagent solutions:

(1) Solution A: Protective colloid gum acasia, 50g in 100ml of distilled water.

(2) Solution B: Citrate buffer 23.5g Tri-Na citrate-2 hydrate and 16.36g anhydrous citric acid in 100ml of distilled water.

(3) Solution C: Reducing agent hydroquinone 0.85g in 15ml of distilled water (prepared just prior to use).

(4) Solution D: Silver lactate 0.11g in 15ml distilled water (prepared just prior to use).

The four solutions were mixed together before use and then stored in the dark. The ratio of each solution in the final enhancer solution was: Solution A - 7.5ml made up to 60ml with distilled water; Solution B - 10ml; Solution C - 15ml; Solution D - 15ml. 100 microlitres of the mixed solution was added to each well in the dark and allowed to incubate for 15 minutes before washing three times with distilled water.

CLAIMS

1. A method for visually enhancing immunogold assays comprising exposing completed immunogold assay tests to a solution comprising silver ions, whereby silver is nucleated onto at least a portion of the gold, and then relating the presence and/or amount of nucleated silver to the presence and/or amount of the target analyte.

2. A method for the determination of the presence or quantity of a target analyte in a fluid comprising:

(a) immobilising on a solid substrate a specific binding agent with which the target analyte will combine specifically

(b) exposing the immobilised specific binding agent to a sample of fluid to be assayed

(c) exposing the immobilised specific binding agent from step (b) to a secondary binding agent capable of binding to the specifically combined target analyte and to which colloidal gold particles have been bound

(d) exposing the immobilised specific binding agent from step (c) to a solution containing silver ions whereby the silver nucleates onto at least a portion of the gold bound to any bound secondary binding reagent and

(e) relating the presence and/or amount of nucleated silver to the presence and/or amount of target analyte in the fluid.

3. A method for the determination of the presence and/or quantity of a target analyte in a fluid comprising:

(a) immobilising on a solid substrate a specific binding agent with which the target analyte will combine specifically

(b) exposing the specific binding agent to a mixture of the fluid with a solution containing a quantity of the target analyte, or an analogue thereof which will specifically bind to said specific binding agent, to which colloidal gold particles have been bound

(c) exposing the immobilised specific binding agent from step (b) to a solution comprising silver ions whereby silver nucleates onto at least a portion of any gold bound to said binding agent via any labelled target analyte or analogue thereof and

(d) relating the presence and/or amount of nucleated silver to the presence and/or amount of target analyte in the fluid.

4. A method for the determination of the presence and/or quantity of a target analyte in a fluid comprises:

(a) immobilising on a solid substrate a target analyte with which a specific binding agent will combine specifically

(b) exposing the immobilised target analyte to a mixture of the fluid to be assayed and the specific binding agent to which said analyte combines specifically

(c) exposing the immobilised target analyte from step (b) to a secondary binding agent capable of binding to the specifically combined specific binding agent and to which colloidal gold particles have been bound

(d) exposing the immobilised target analyte from step (c) to a



solution comprising silver ions whereby silver nucleates onto at least a portion of the gold bound to any bound secondary binding agent and

(e) relating the presence and/or amount of nucleated silver to the presence and/or amount of target analyte in the fluid.

5. A method according to any one of Claim 1, 2, 3 or 4 wherein the target analyte comprises an antibody, protein, virus, hormone, drug, nucleic acid or polysaccharide.

6. A method according to Claim 1 wherein the target analyte is an immunoglobulin specific for HTLV-I, HIV, Hepatitis, Measles, Chicken Pox, Leukaemia or Rubella;

7. A method according to any one of Claims 1 to 6 wherein the fluid is whole blood, blood serum, blood plasma, semen, tears, urine, sweat, saliva, cerebro-spinal fluid, vaginal secretions, in-vitro fluids, plant fluids or an environmental fluid.

8. A method according to any one of the preceeding claims wherein the target analyte is a specific antibody and the immobilised binding agent is an antigen for which the said antibody is specific.

9. A method according to any of the Claims 1, 4, 5 or 7 wherein the target analyte is a drug and the specific binding reagent used is an antibody specific for it.

10.A method according to any one of the above claims wherein the immobilised specific binding agent or immobilised target analyte comprises a microtitre plate, a microwell strip or a porous membrane coated with said agent or analyte.

11.A method according to any one of the preceeding claims wherein the gold labelled binding agent is an anti-human, anti-rat, anti-mouse, anti-guinea pig or anti-rabbit antibody directed to IgA, IgG, IgM or IgM immunoglobulins.

12.A method according to any of the above claims wherein the silver ion containing solution comprises IntenSE\*II, IntenSe M, IntenSE BL, Sigma SE-100, silver lactate, silver citrate, silver nitrate or silver chloride reagents.

13.A method according to Claim 12 wherein the concentration of the silver ions in the solution is from 0.1 to 1000 mM.

14.A method according to Claim 12 wherein the concentration of the silver ions in the solution is from 1 to 100 mM.

15.A method according to Claim 12 wherein the concentration of the silver ions in the solution is from 2 to 20 mM.

16.A method according to Claim 12 wherein the silver ion containing solution also comprises a stabiliser and/or a retardant.

17.A method according to Claim 16 wherein the stabiliser comprises a reducing agent.

18.A method according to Claim 17 wherein the reducing agent is a hydroquinone.

19.A method according to Claim 16 wherein the retardant comprises gum acasia.

20. A method according to Claim 18 wherein the hydroquinone is used at a concentration between 1% and 0.01% by weight.

21. A method according to Claim 19 wherein the gum acasia is used at a concentration of between 1% and 0.01% by weight.

22.A method according to any previous claim wherein the colloidal gold is 10nm or less in particle size.

23.A method according to Claim 22 wherein the colloidal gold is 5nm in particle size.

24.A method according to any one of Claim 1, 2, 3 or 4 wherein the gold labelled binding agent is incubated with the immobilised combined unlabelled agents for a period of from 10 to 60 minutes.

25.A method according to Claim 24 wherein the gold labelled binding agent is incubated with the immobilised combined unlabelled agents for a period of from 20 to 40 minutes.

26.A method according to Claim 24 wherein the gold labelled binding agent is incubated with the immobilised combined unlabelled agents for about 30 minutes.

27.A method according to one of Claims 1, 2, 3 or 4 wherein the silver solution is incubated with the completed immunogold assay for between 10 and 60 minutes.

28. A method according to Claim 27 wherein the silver solution is incubated with the completed immunogold assay for between 20 and 30 minutes.

29. A method according to Claim 1, 2, 3 or 4 wherein the immobilised agents are washed between each exposure.

30.A method according to Claim 29 wherein the washing is carried out using phosphate buffer saline or demineralised water.

31.A method according to Claim 1, 2, 3 or 4 wherein the amount of target analyte is determined by reading the absorbance at a specific wavelength of the completed silver enhanced immunogold assay surface and then relating that to the absorbance for a standard amount of analyte.

32. An assay kit comprising:

(a) specific binding agent with which a target analyte will combine specifically immobilised onto a solid substrate

(b) a secondary binding agent capable of binding to the

combined target antibody and to which colloidal gold particles have been bound and

(c) a solution comprising silver ions

33. An assay kit comprising:

(a) a specific binding agent with which a target analyte will combine specifically immobilised onto a solid substrate

(b) target analyte, or a specifically binding analogue thereof, to which colloidal gold particles have been bound and

(c) a solution comprising silver ions

34. An assay kit comprising:

(a) a target analyte with which a specific binding agent will combine specifically immobilised onto a solid substrate

(b) said specific binding agent

(c) a secondary binding agent to which colloidal gold particles have been bound and which is capable of binding to the specific binding agent when that is bound to the immobilised target analyte.

(d) a solution comprising silver ions.

35. A kit according to any one of Claims 32, 33 or 34 further comprising a buffer solution and a solution of demineralised water.

36. A kit according to any one of the Claims 32 to 35 wherein the silver ion comprising solution comprises a soluble salt of silver.

37. A kit according to Claim 36 wherein the silver ion comprises a soluble organic silver salt.

38. A kit according to any one of Claims 32 to 34 wherein there is further included a stabiliser and/or a retardant for the silver ion comprising solution.

39. A kit according to Claim 38 wherein said stabiliser comprises a reducing reagent.

40. A kit according to Claim 39 wherein said reducing agent comprises a hydroquinone.

41. A kit according to Claim 38 wherein the retardant comprises gum acasia.

42. A kit according to any one of the Claims 32 to 34 wherein the silver ion comprising solution comprises silver lactate, silver citrate, silver nitrate, silver chloride or the constituents of the IntenSE\*II, IntenSE M, IntenSE BL or Sigma SE-100 reagents.

43. A method according to any one of Claims 1 to 31 as herein described with reference to the Examples 1 to 8.

44. A kit according to any one of the Claims 32 to 42 comprising reagents and microtitre wells as described for the SEGLISA procedures of any one of the Examples 1 to 8.

45. A method according to any one of the Claims 1, 2 or 5 wherein the presence of a silver deposit indicates the presence of the target analyte in the fluid.

46. A method according to any one of the Claim 3 or Claim 4 or Claims 5 wherein the absence of a silver deposit indicates the presence of the target analyte in the fluid.

47. A method according to any one of the Claims 1 to 31, 43, 45, or 46 wherein the target analyte comprises an antibody to HIV or rubella or comprises theophylline or human growth hormone.

48. A kit according to any one of the Claims 32 to 42 or 44 wherein specific binding agent for an antibody to HIV or rubella or specific binding agent for theophylline or human growth hormone is included.



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Fig.1.

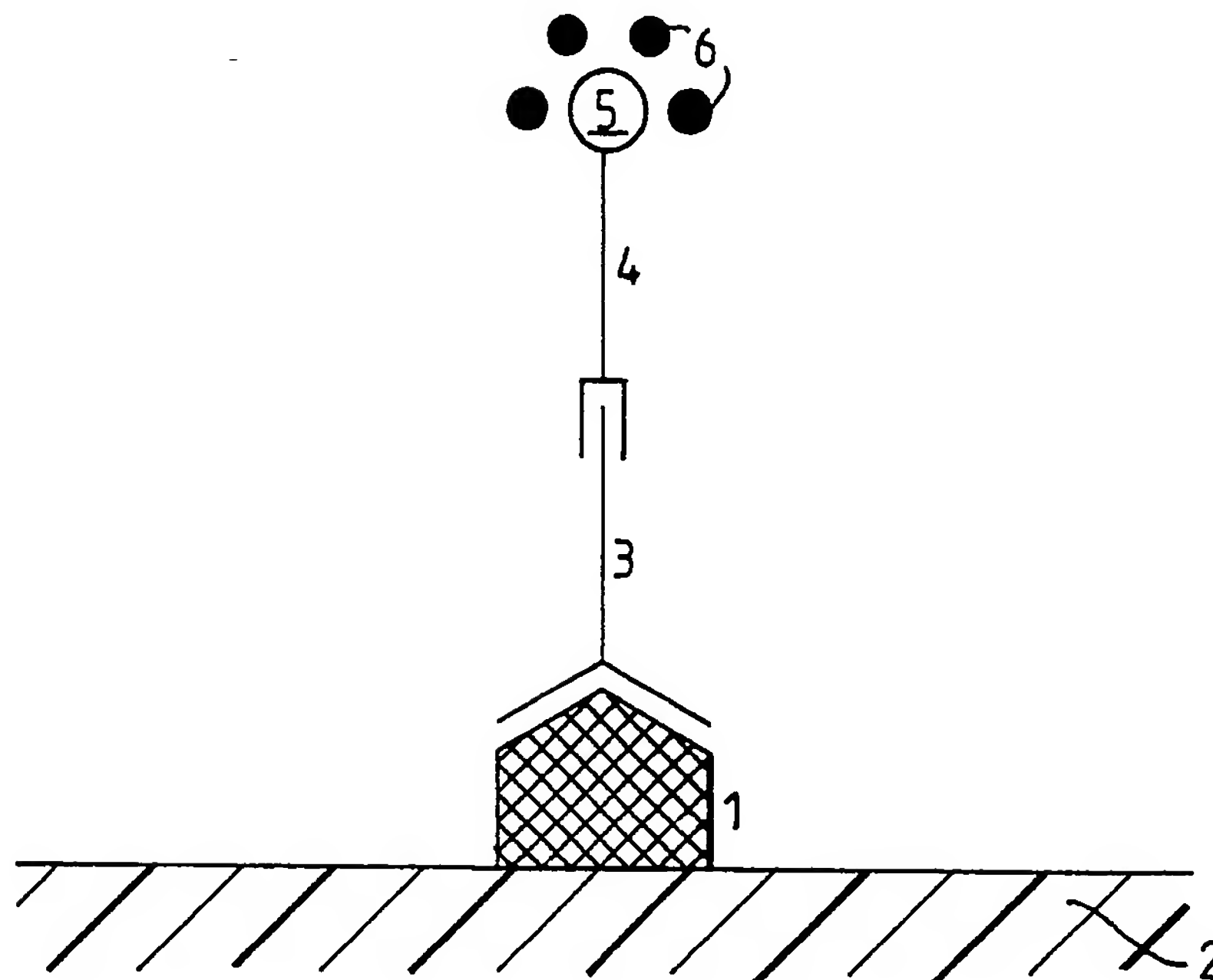
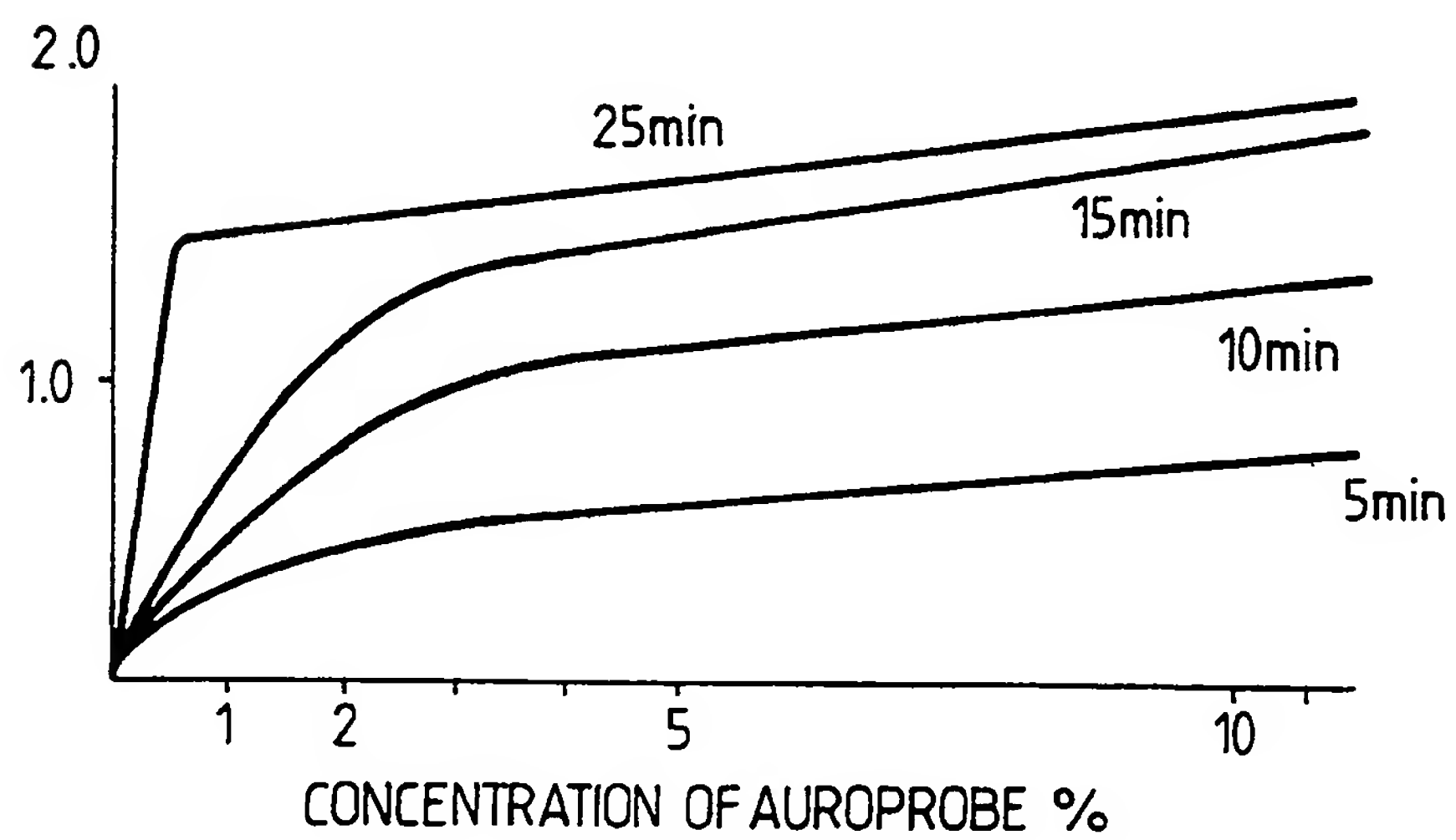


Fig.2.



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Fig.3.

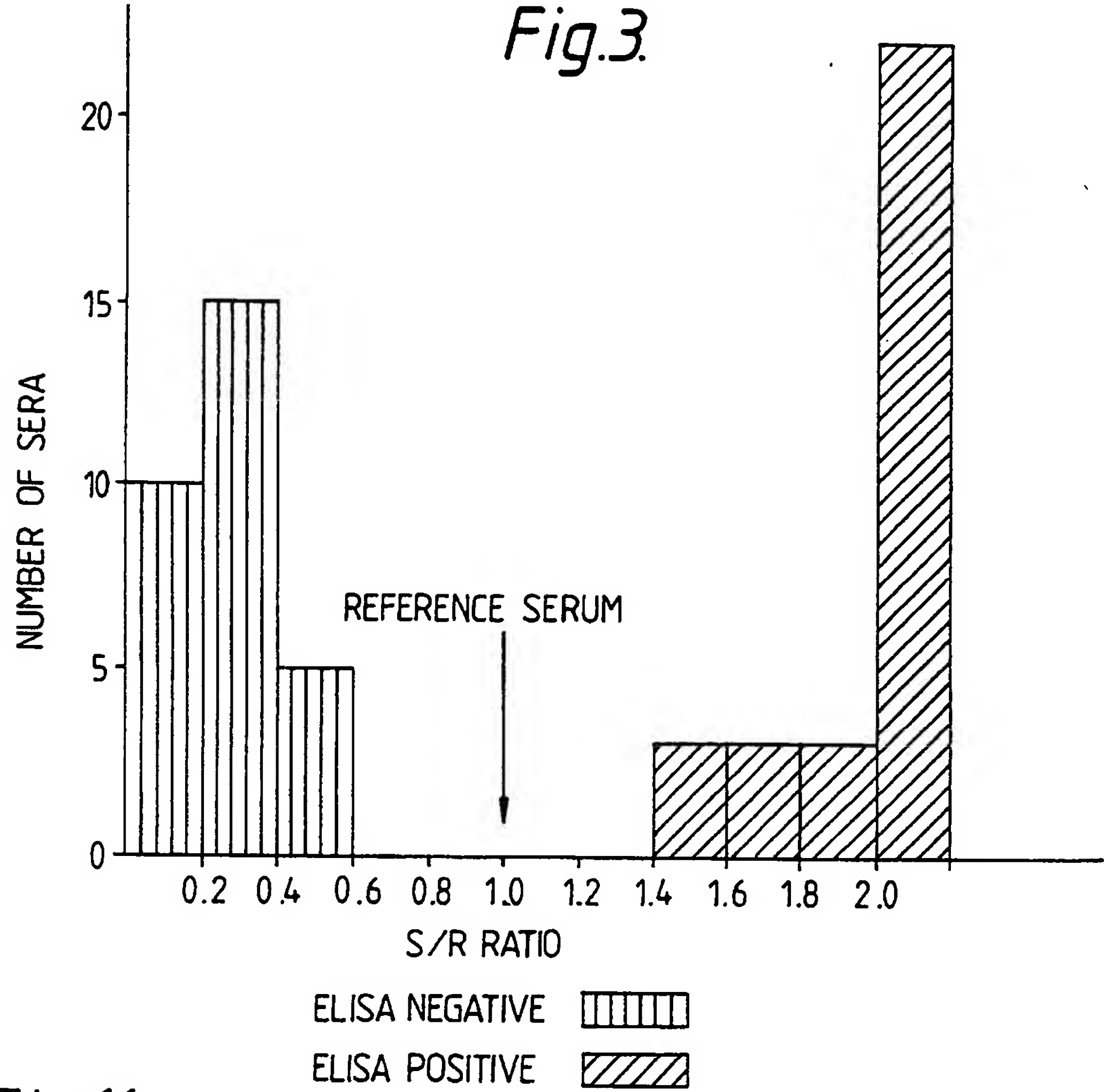
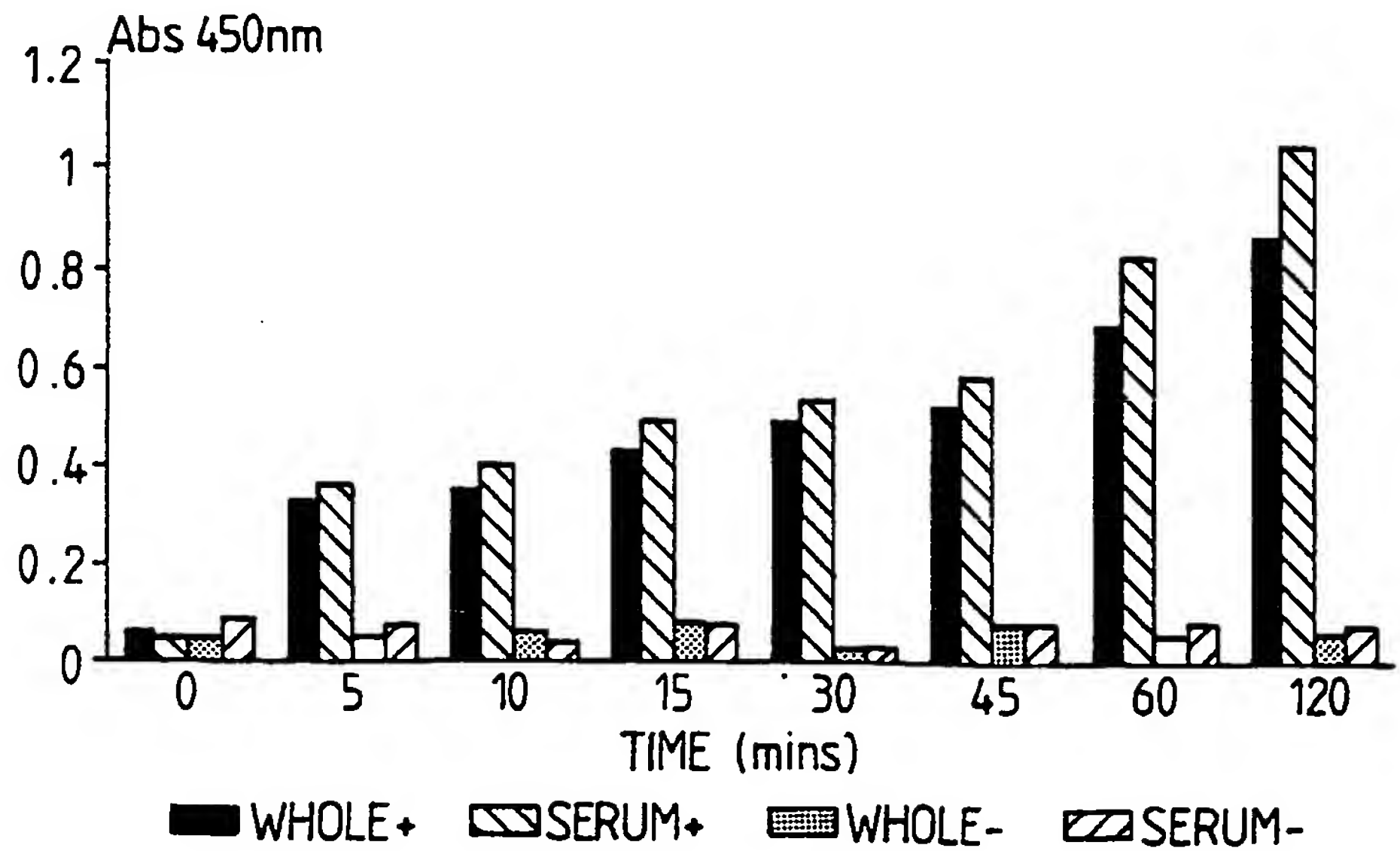
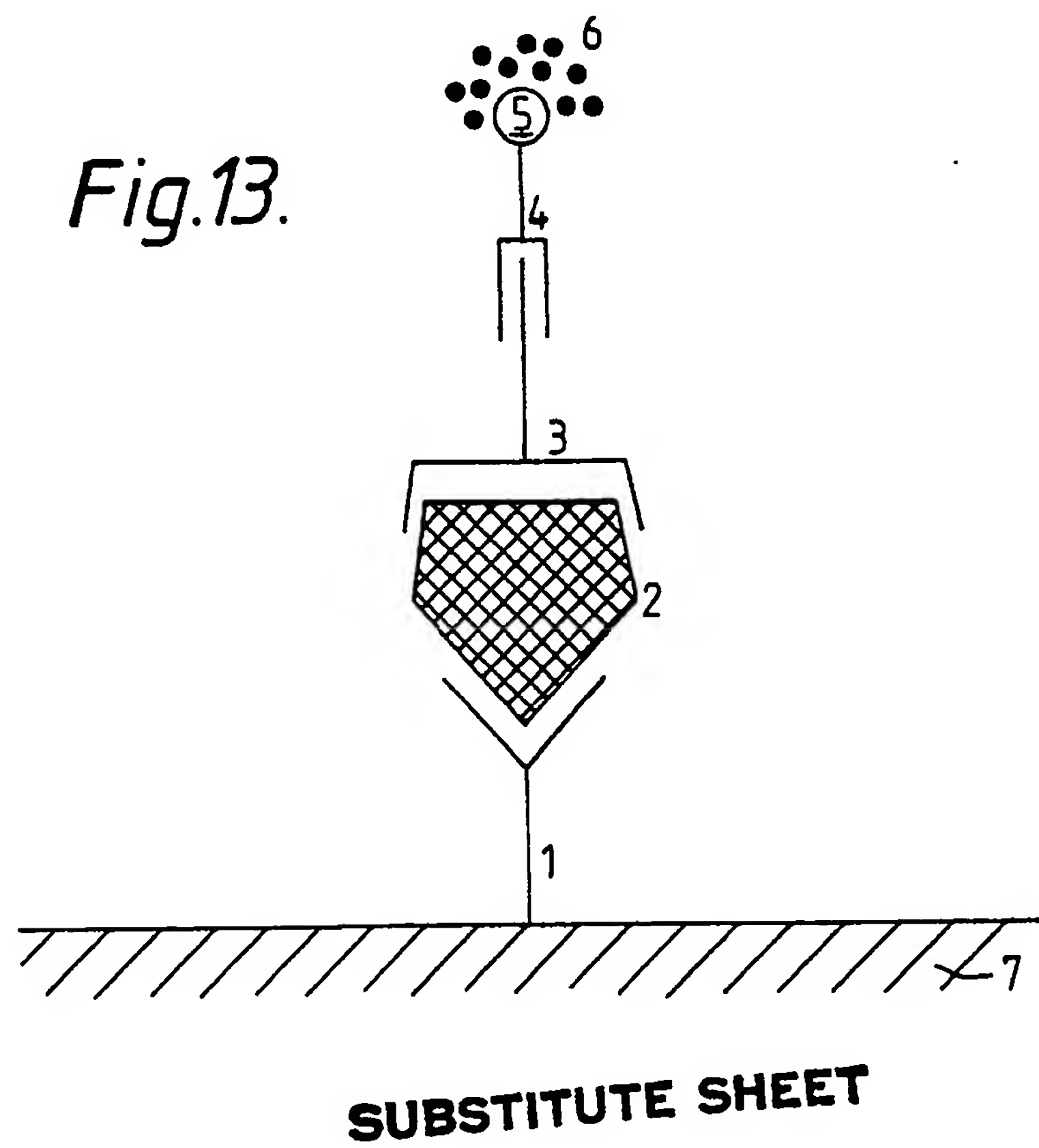
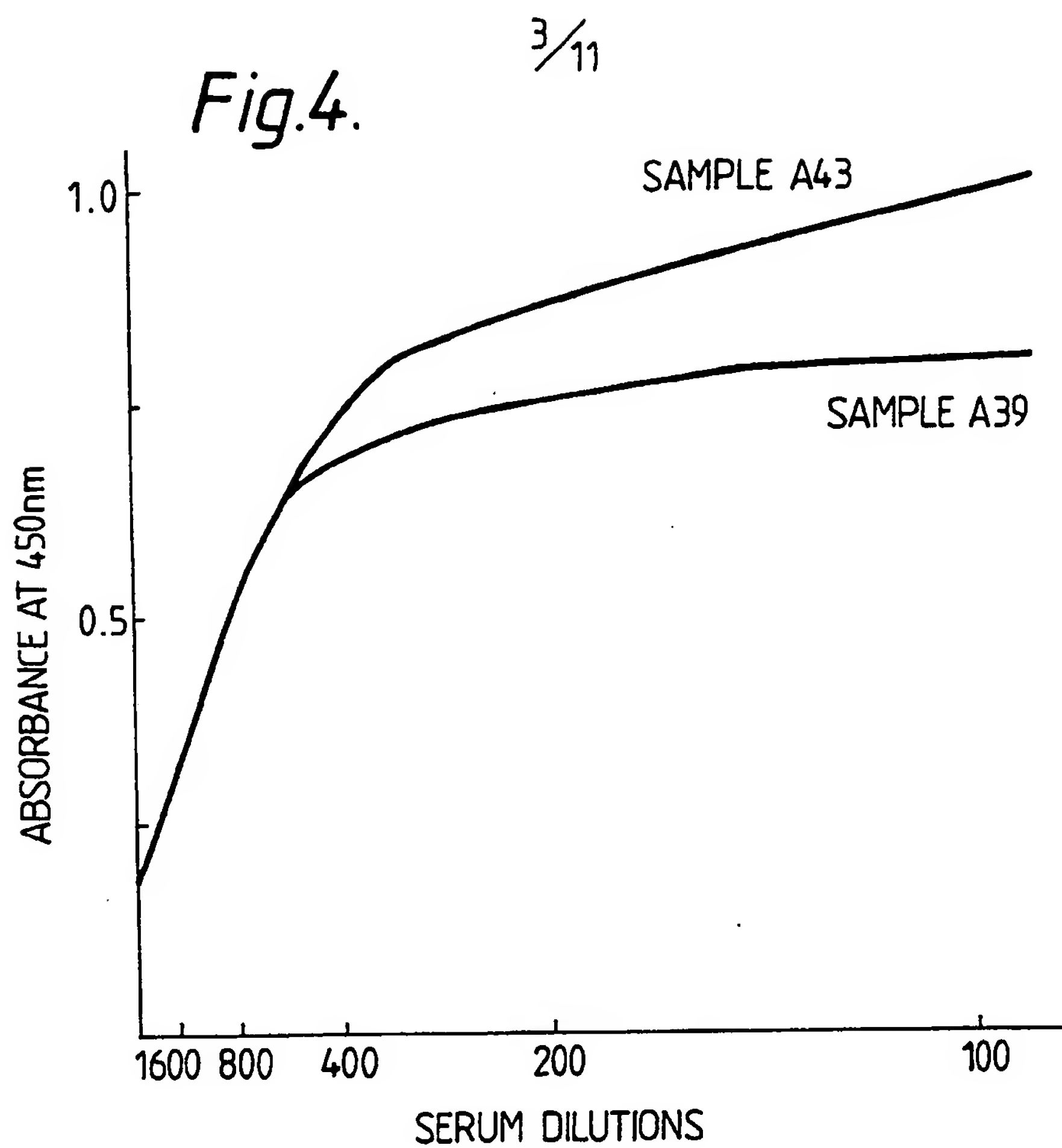
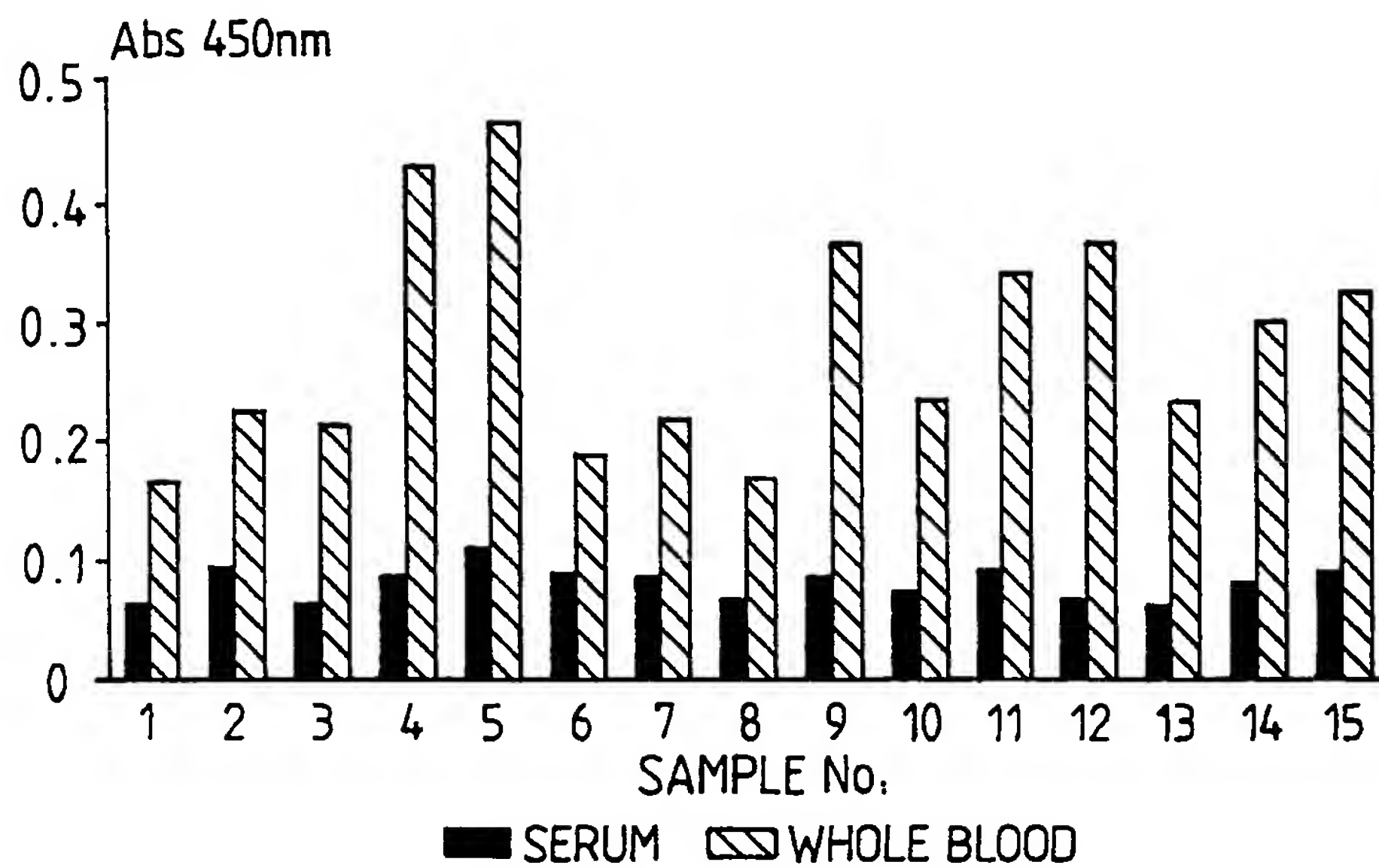
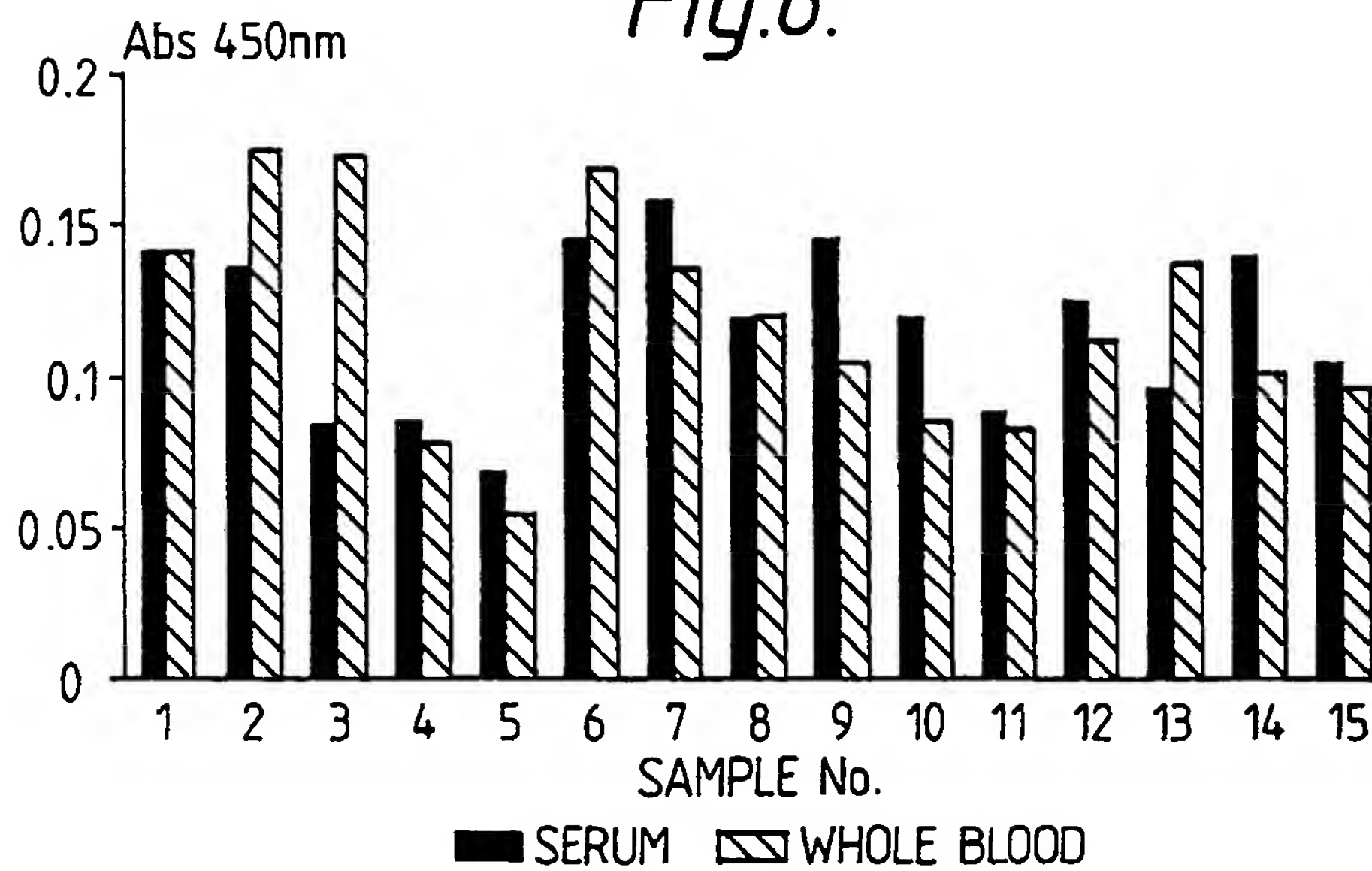


Fig.11.

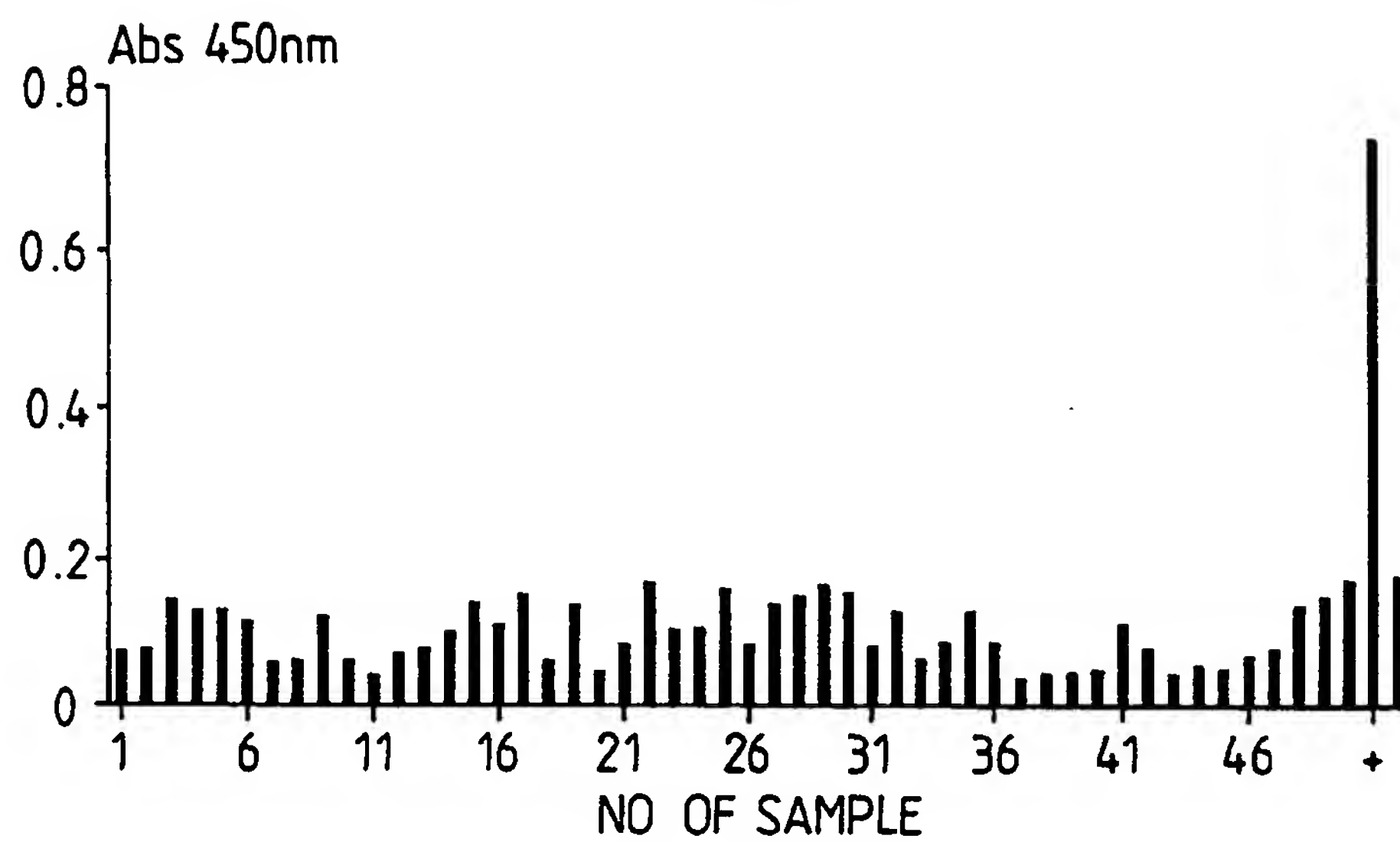
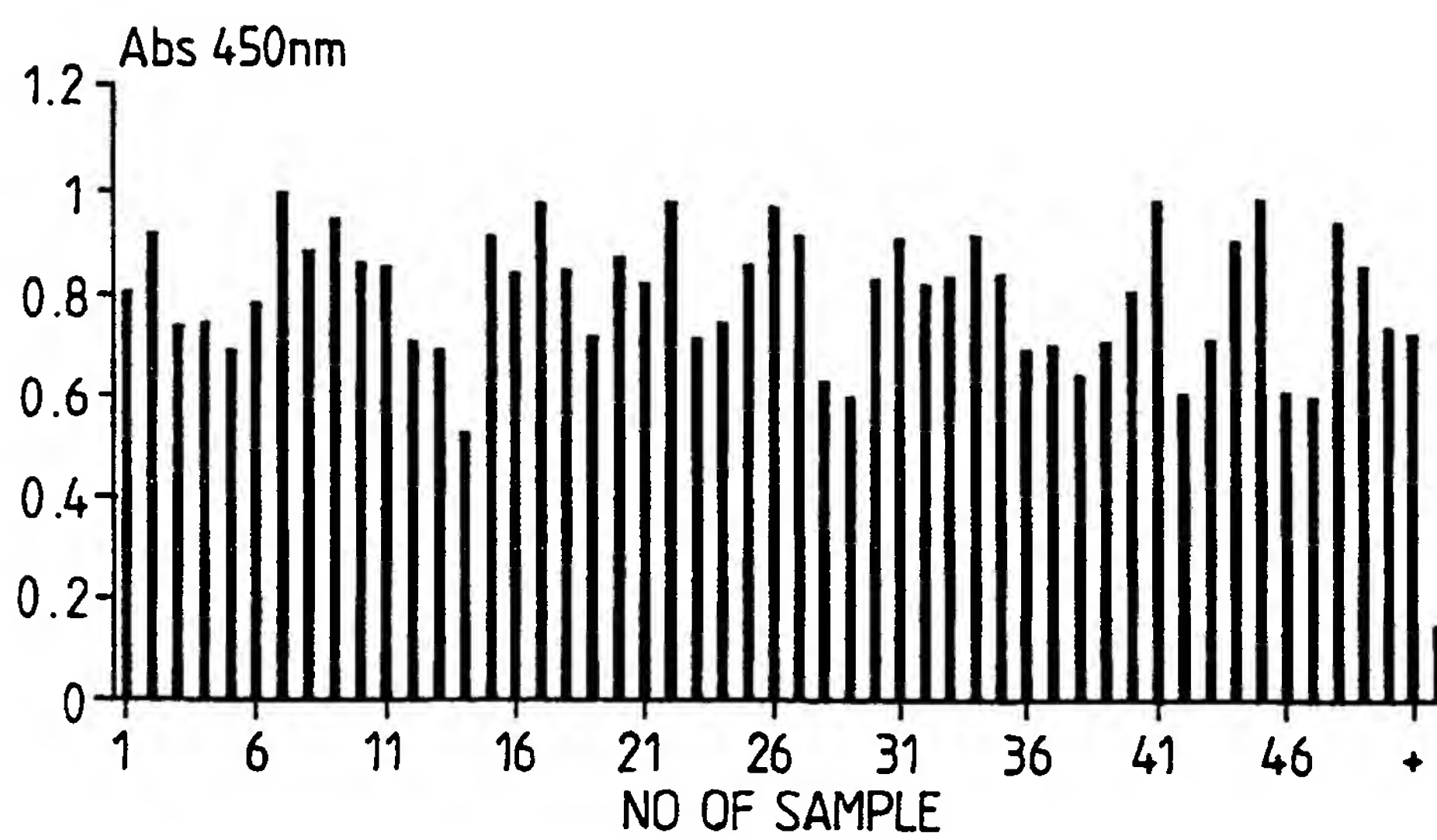




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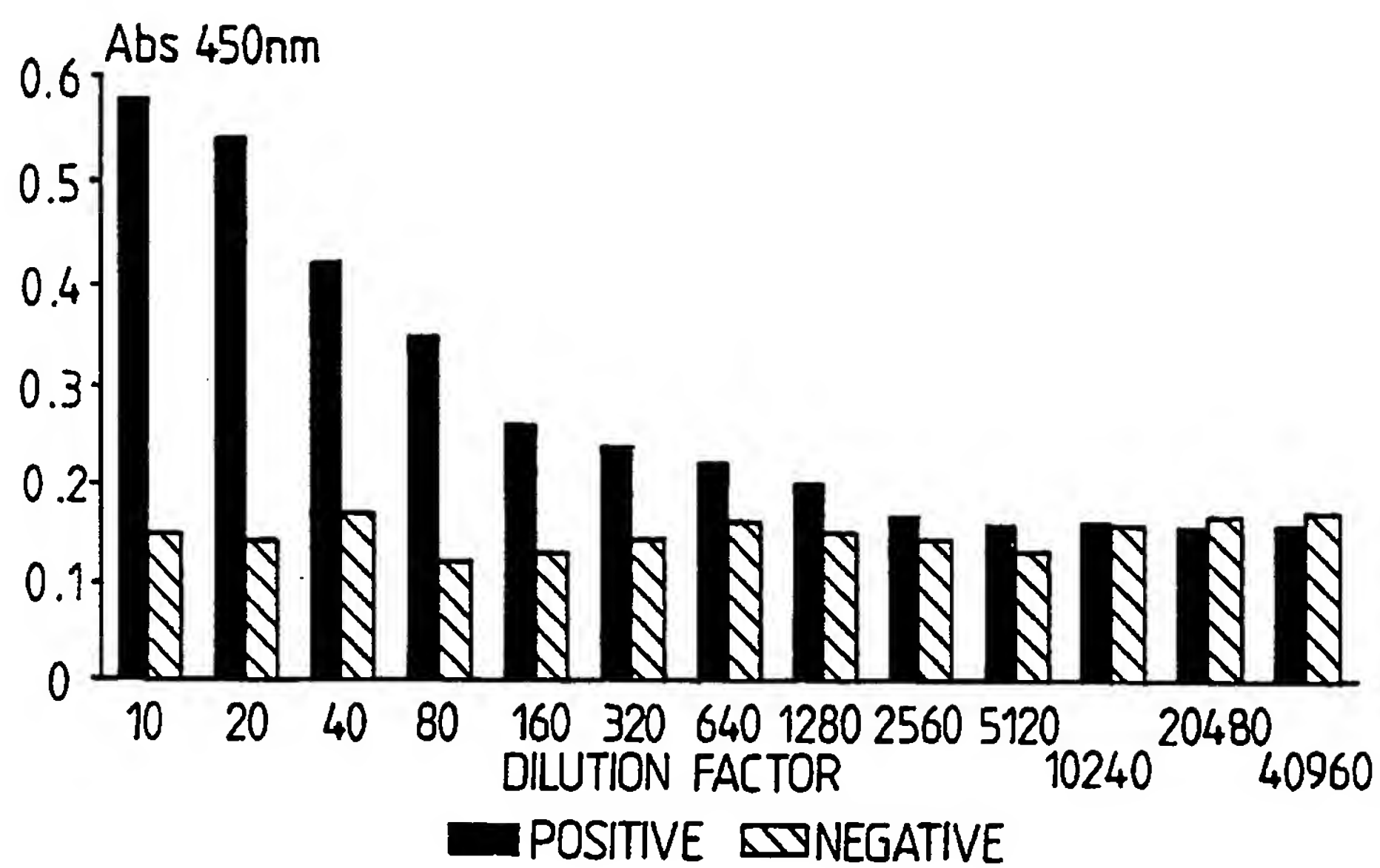
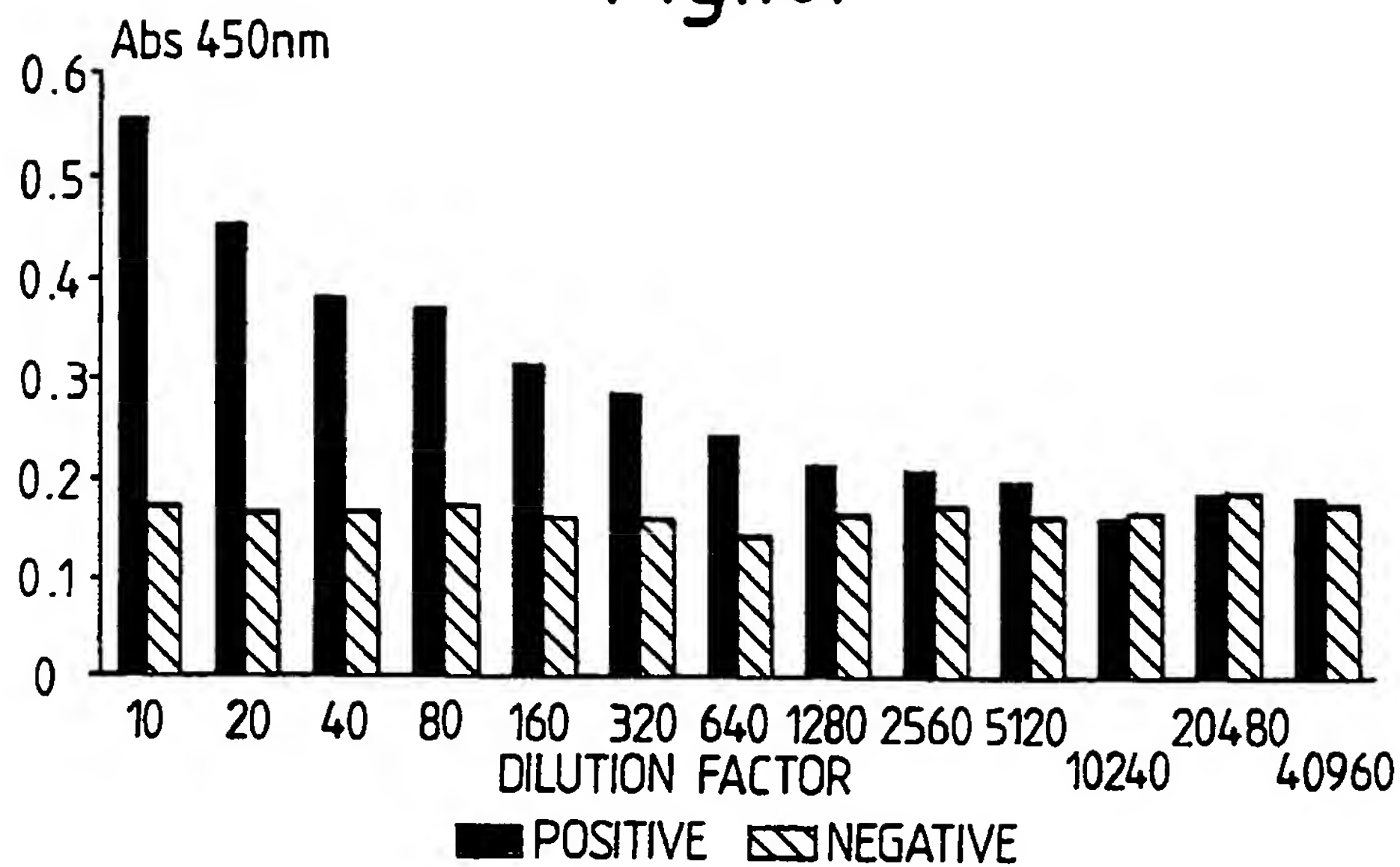
*Fig.5.**Fig.6.***SUBSTITUTE SHEET**

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*Fig. 7.**Fig. 8.*

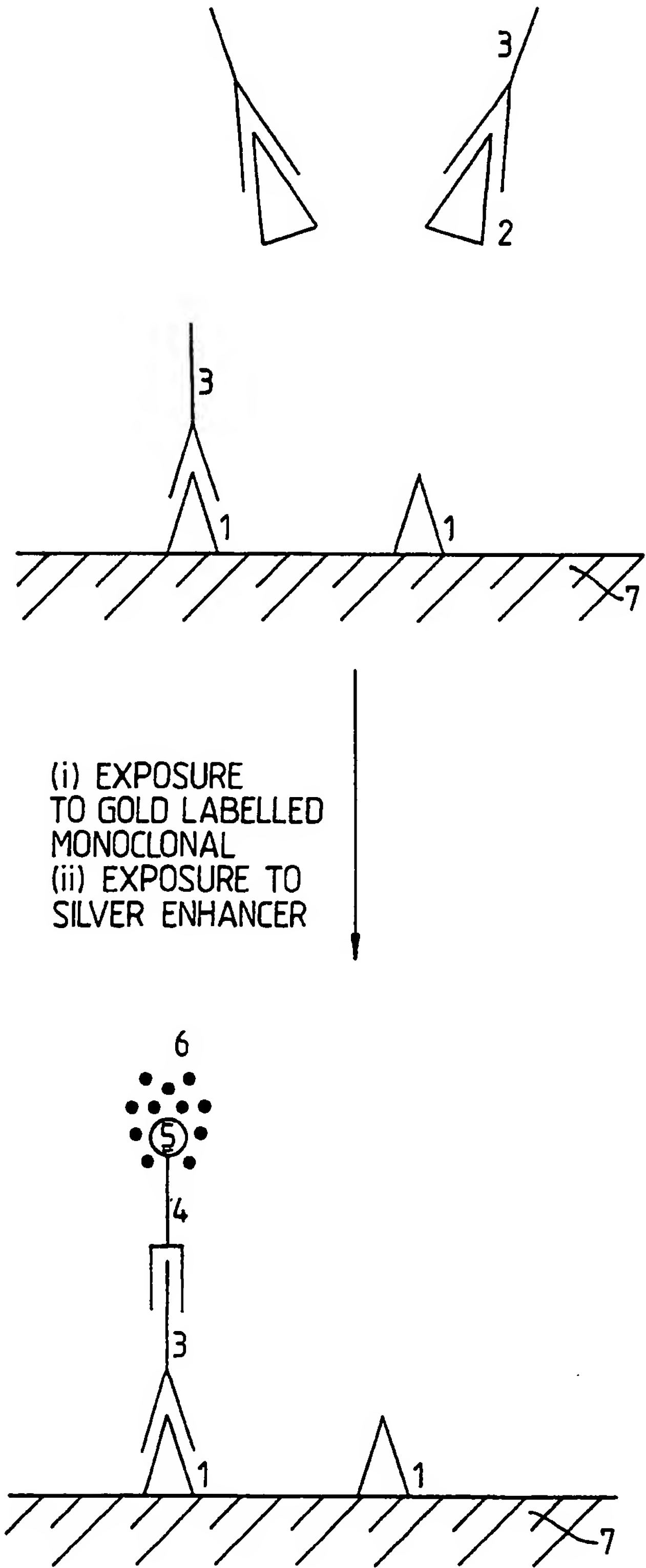
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*Fig.9.**Fig.10.***SUBSTITUTE SHEET**

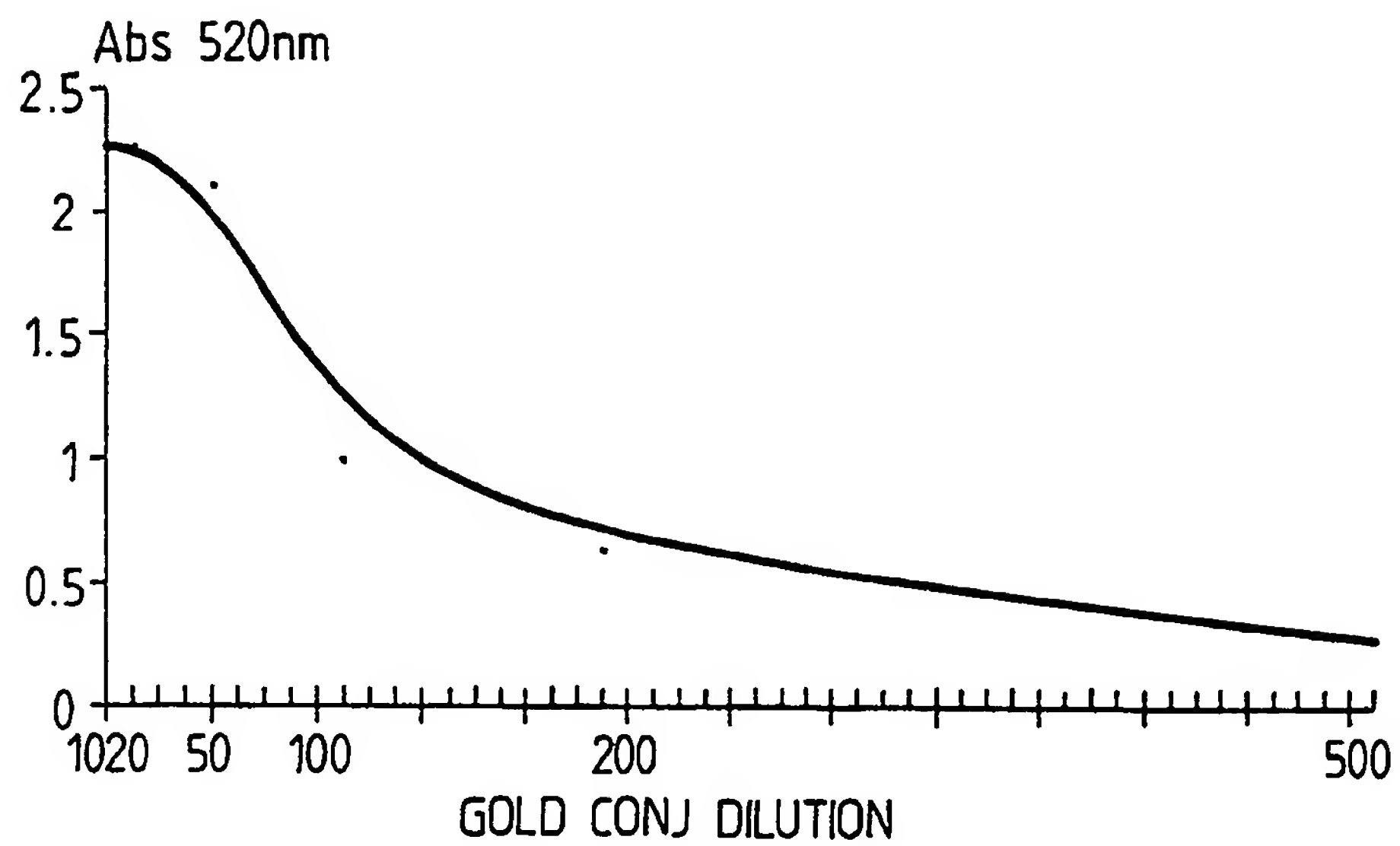
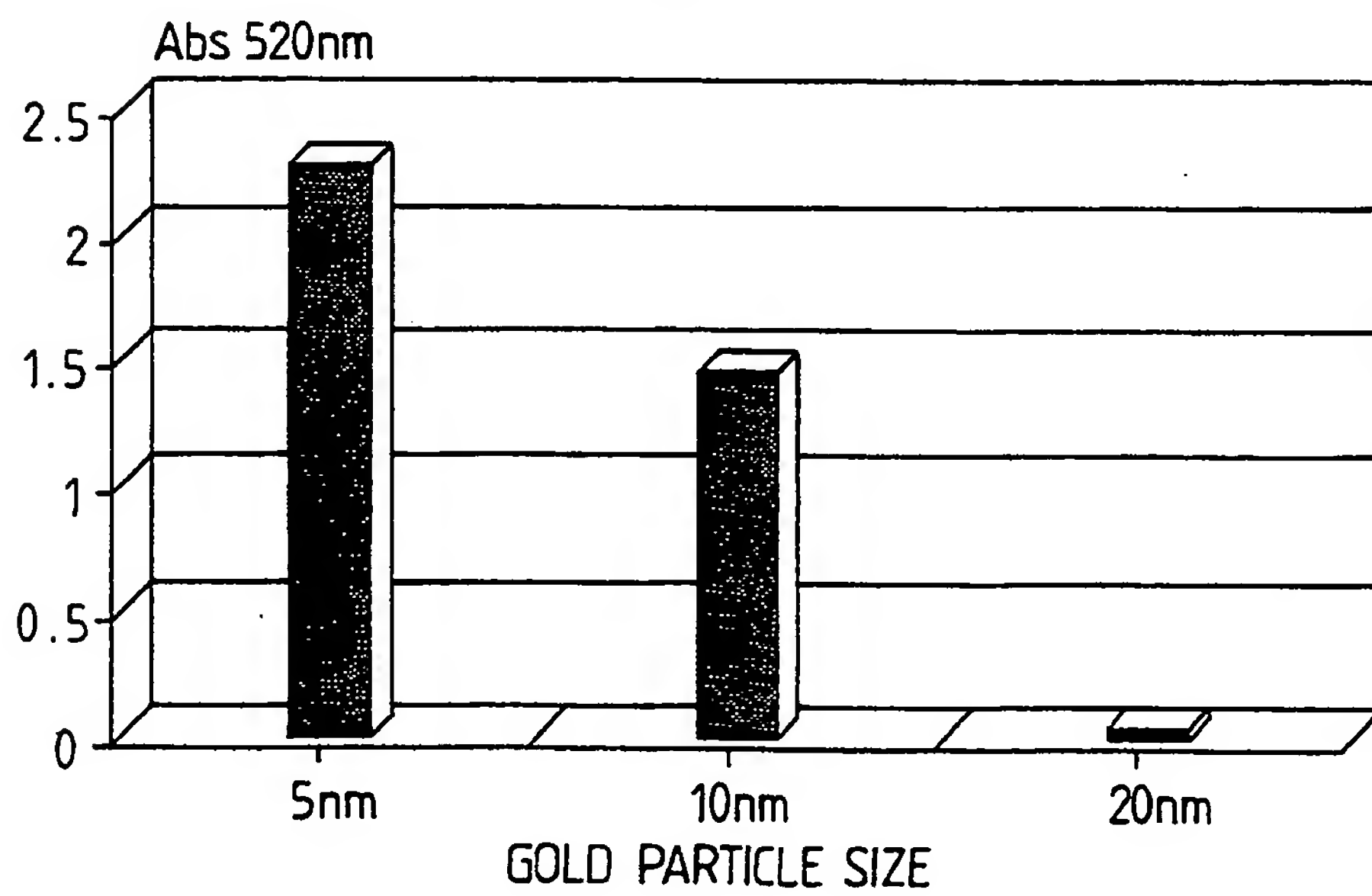
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Fig.12.

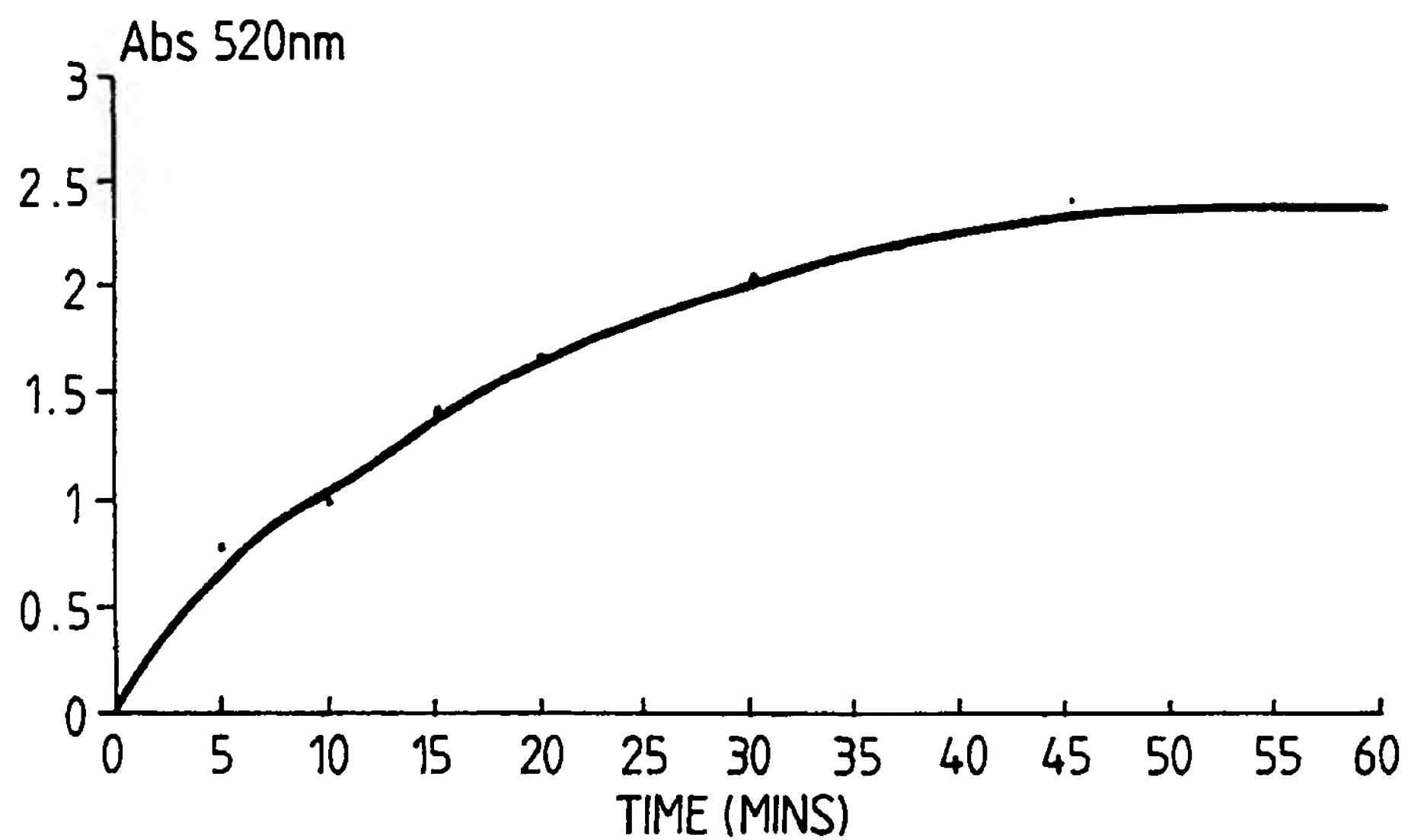
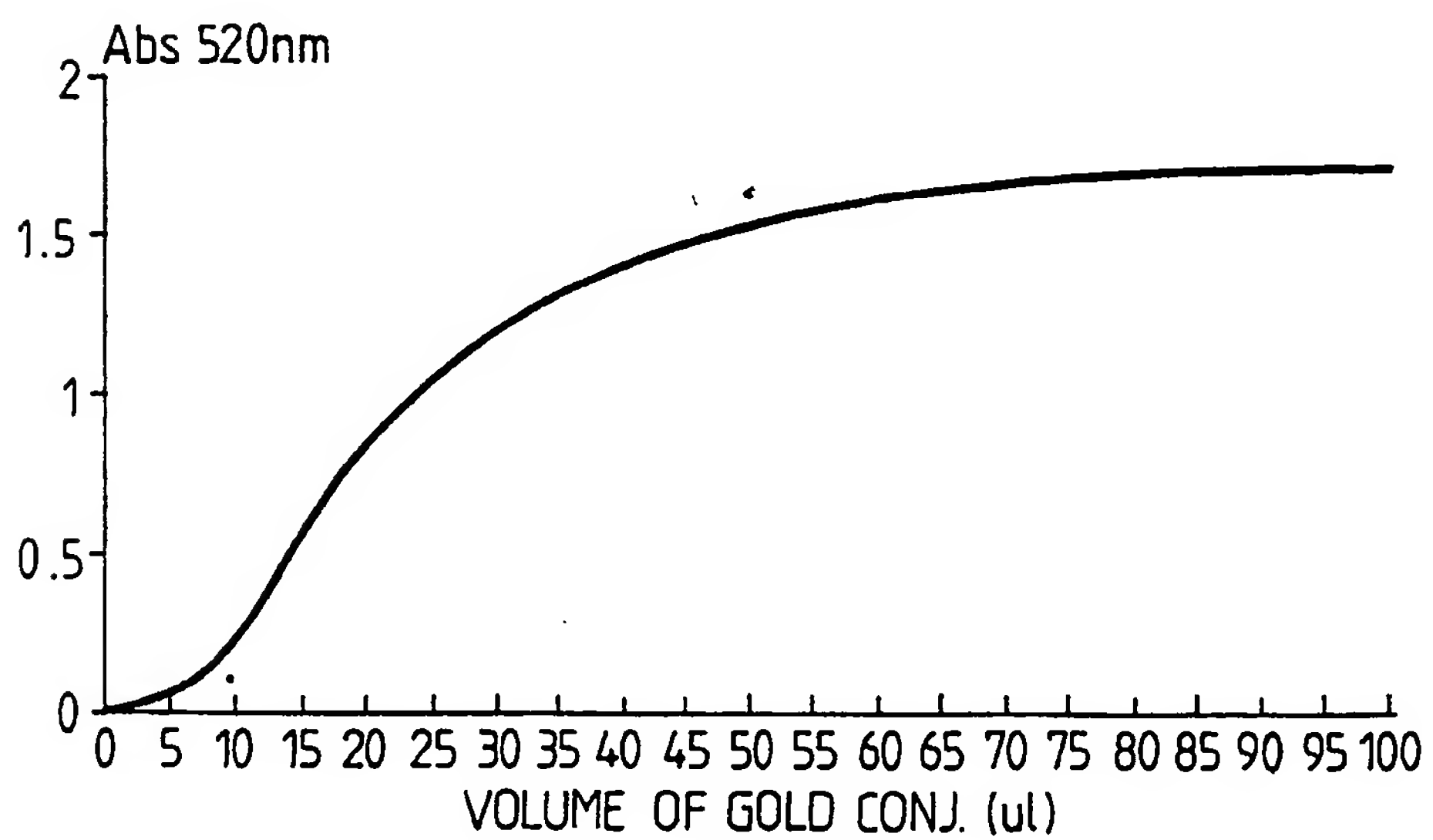




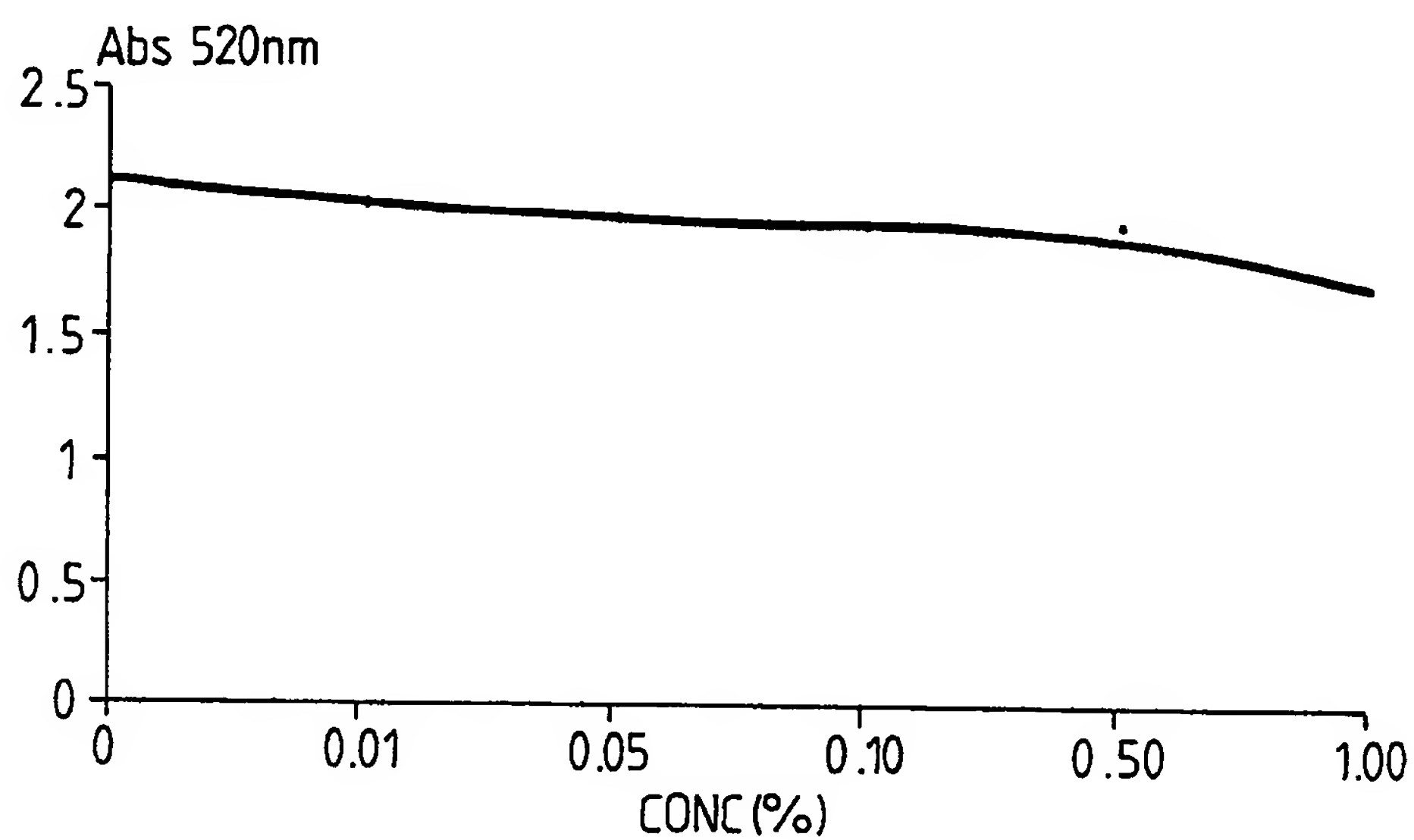
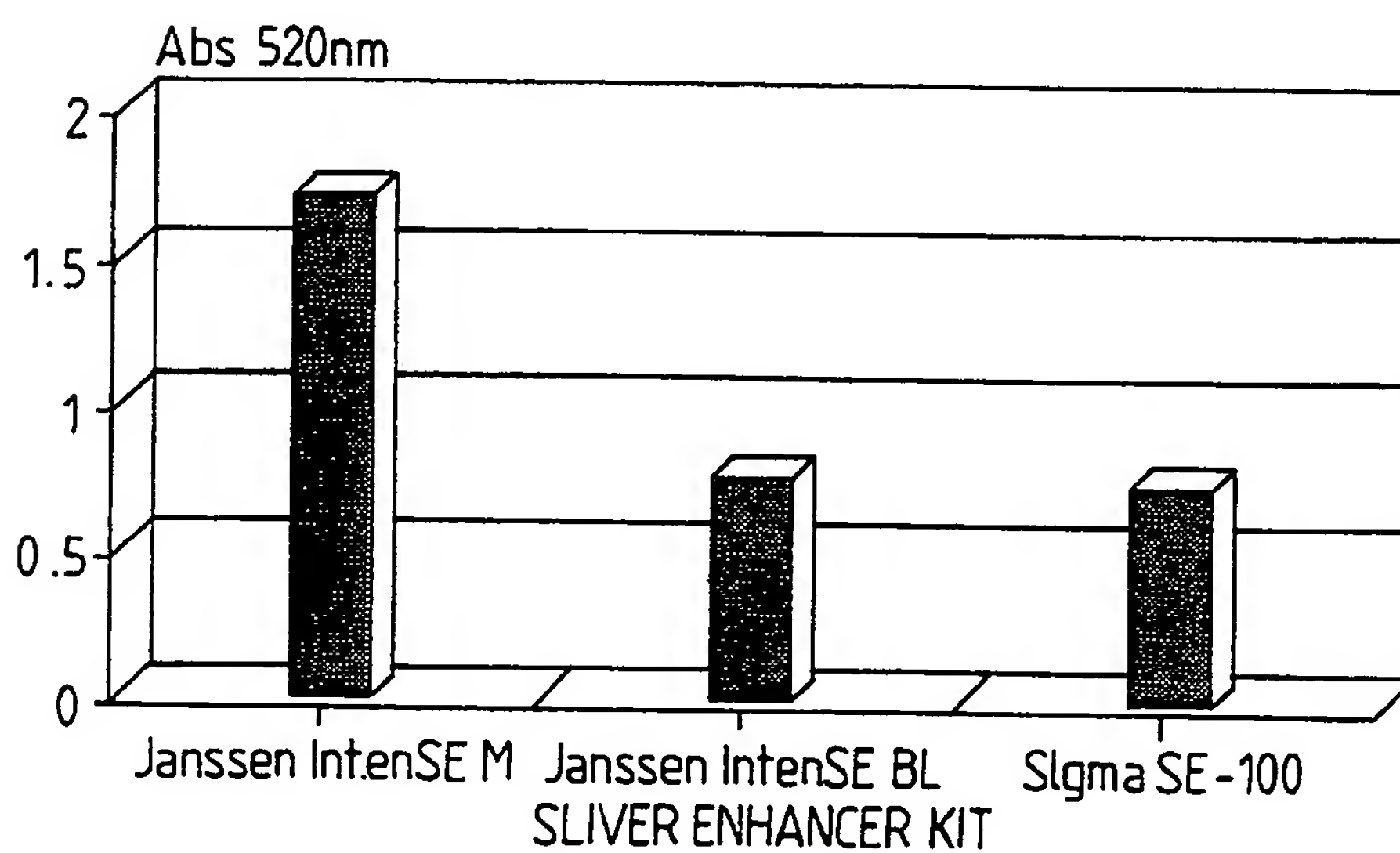
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*Fig.14.**Fig.15.***SUBSTITUTE SHEET**

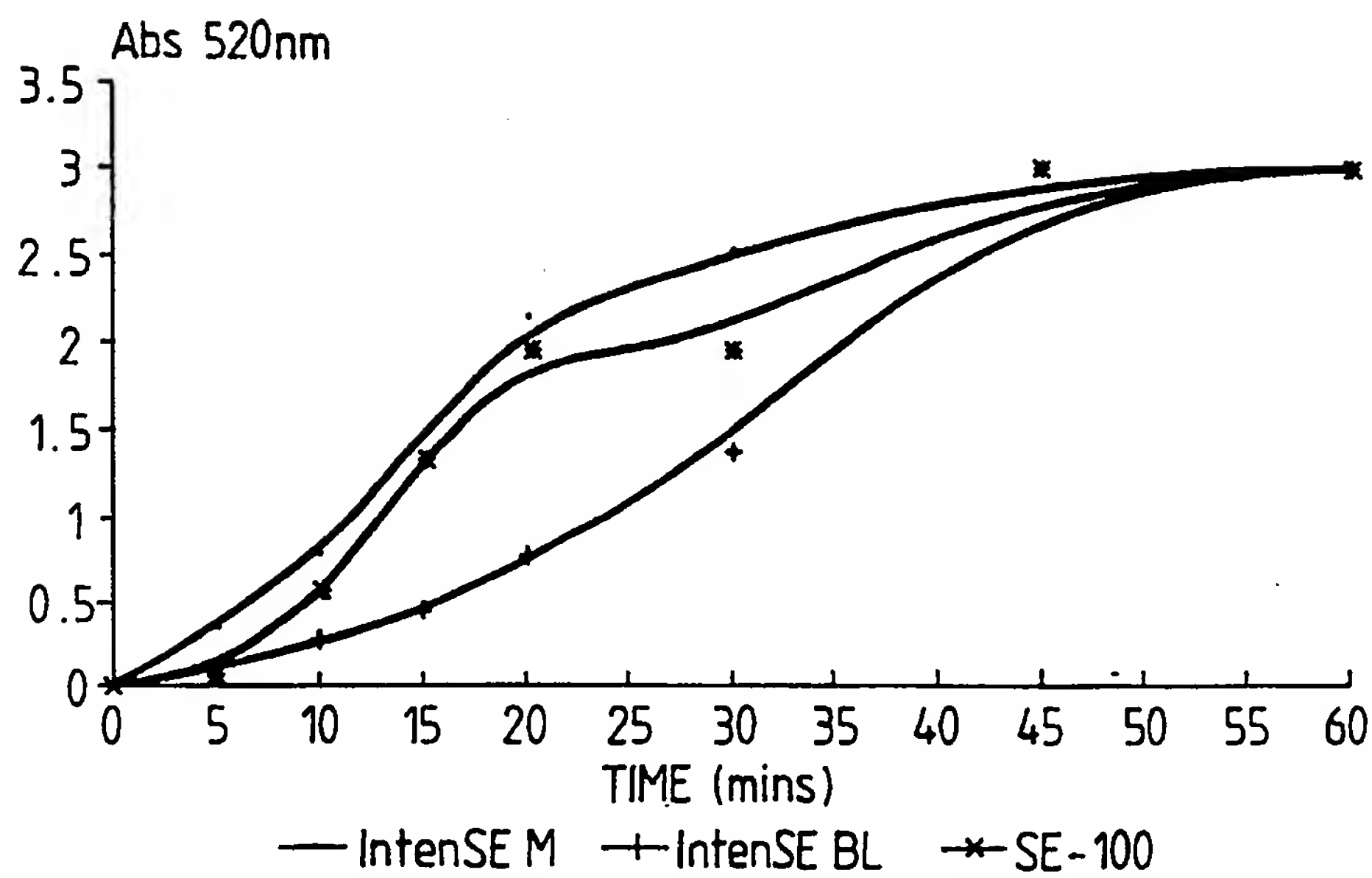
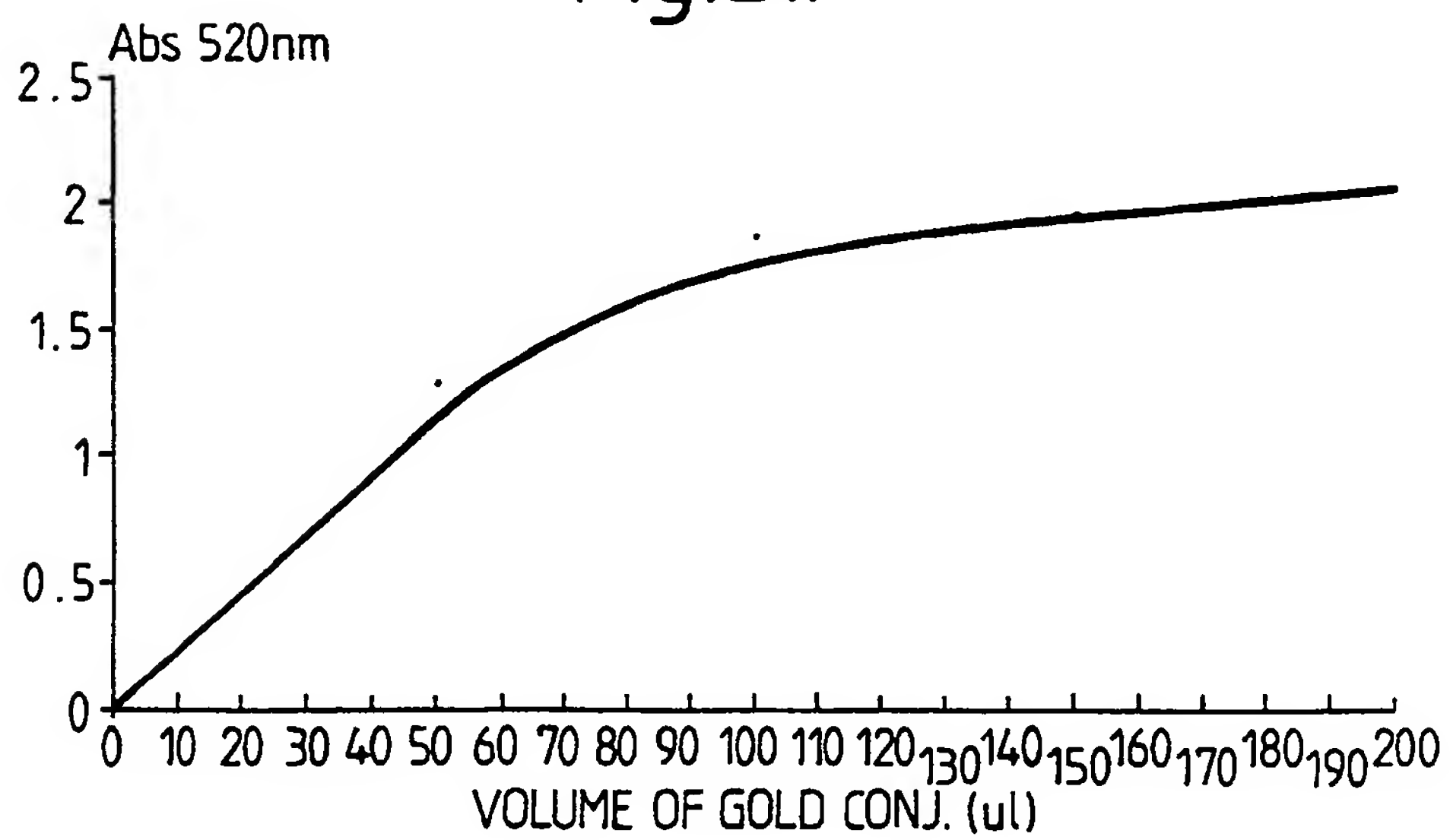
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*Fig.16.**Fig.17.***SUBSTITUTE SHEET**

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*Fig.18.**Fig.19.***SUBSTITUTE SHEET**

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*Fig.20.**Fig.21.***SUBSTITUTE SHEET**

# INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/01046

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC IPC <sup>5</sup> : G 01 N 33/53, 33/543, 33/94, 33/569, 33/74											
<b>II. FIELDS SEARCHED</b> <div style="text-align: right; font-size: small;">Minimum Documentation Searched <sup>7</sup></div> <table style="width: 100%; border: none;"> <tr> <td style="width: 30%; border: none; vertical-align: top;"> <div style="border-bottom: 1px solid black; padding-bottom: 5px;">Classification System</div> <div style="padding: 5px 0 0 0;">IPC<sup>5</sup></div> </td> <td style="width: 70%; border: none; vertical-align: top;"> <div style="border-bottom: 1px solid black; padding-bottom: 5px;">Classification Symbols</div> <div style="padding: 5px 0 0 0;">G 01 N</div> </td> </tr> </table> <div style="text-align: center; font-size: x-small; margin-top: 10px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup></div>			<div style="border-bottom: 1px solid black; padding-bottom: 5px;">Classification System</div> <div style="padding: 5px 0 0 0;">IPC<sup>5</sup></div>	<div style="border-bottom: 1px solid black; padding-bottom: 5px;">Classification Symbols</div> <div style="padding: 5px 0 0 0;">G 01 N</div>							
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<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; font-size: x-small;">Category <sup>9</sup></th> <th style="width: 70%; font-size: x-small;">Citation of Document, <sup>11</sup> with Indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 20%; font-size: x-small;">Relevant to Claim No. <sup>13</sup></th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td style="vertical-align: top;">           EP, A, 0293947 (JANSSEN PHARMACEUTICA N.V.) 7 December 1988            see column 13, lines 35-50; column 16, lines 11-21; column 17, lines 35-55; column 20, lines 13-21; claim 15            --         </td> <td style="text-align: center; vertical-align: top;">1-48</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td style="vertical-align: top;">           EP, A, 0158746 (JANSSEN PHARMACEUTICA N.V.) 23 October 1985            see page 7, lines 6-23; page 10, lines 18-30; page 21, line 29 - page 22, line 10            -----         </td> <td style="text-align: center; vertical-align: top;">1-48</td> </tr> </tbody> </table>			Category <sup>9</sup>	Citation of Document, <sup>11</sup> with Indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	X	EP, A, 0293947 (JANSSEN PHARMACEUTICA N.V.) 7 December 1988 see column 13, lines 35-50; column 16, lines 11-21; column 17, lines 35-55; column 20, lines 13-21; claim 15 --	1-48	X	EP, A, 0158746 (JANSSEN PHARMACEUTICA N.V.) 23 October 1985 see page 7, lines 6-23; page 10, lines 18-30; page 21, line 29 - page 22, line 10 -----	1-48
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X	EP, A, 0293947 (JANSSEN PHARMACEUTICA N.V.) 7 December 1988 see column 13, lines 35-50; column 16, lines 11-21; column 17, lines 35-55; column 20, lines 13-21; claim 15 --	1-48									
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<div style="display: flex; justify-content: space-between; font-size: x-small;"> <div style="width: 48%;"> <p><b>* Special categories of cited documents: <sup>10</sup></b></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 48%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Δ" document member of the same patent family</p> </div> </div>											
<b>IV. CERTIFICATION</b> <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none; vertical-align: top;"> <div style="border-bottom: 1px solid black; padding-bottom: 5px;">Date of the Actual Completion of the International Search</div> <div style="padding: 5px 0 0 0;">1st October 1990</div> </td> <td style="width: 50%; border: none; vertical-align: top;"> <div style="border-bottom: 1px solid black; padding-bottom: 5px;">Date of Mailing of this International Search Report</div> <div style="padding: 5px 0 0 0;">23. 10. 90</div> </td> </tr> <tr> <td style="width: 50%; border: none; vertical-align: top;"> <div style="border-bottom: 1px solid black; padding-bottom: 5px;">International Searching Authority</div> <div style="padding: 5px 0 0 0;">EUROPEAN PATENT OFFICE</div> </td> <td style="width: 50%; border: none; vertical-align: top;"> <div style="border-bottom: 1px solid black; padding-bottom: 5px;">Signature of Authorized Officer</div> <div style="padding: 5px 0 0 0;">R.J. Eernisse </div> </td> </tr> </table>			<div style="border-bottom: 1px solid black; padding-bottom: 5px;">Date of the Actual Completion of the International Search</div> <div style="padding: 5px 0 0 0;">1st October 1990</div>	<div style="border-bottom: 1px solid black; padding-bottom: 5px;">Date of Mailing of this International Search Report</div> <div style="padding: 5px 0 0 0;">23. 10. 90</div>	<div style="border-bottom: 1px solid black; padding-bottom: 5px;">International Searching Authority</div> <div style="padding: 5px 0 0 0;">EUROPEAN PATENT OFFICE</div>	<div style="border-bottom: 1px solid black; padding-bottom: 5px;">Signature of Authorized Officer</div> <div style="padding: 5px 0 0 0;">R.J. Eernisse </div>					
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# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

GB 9001046  
SA 38246

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 16/10/90. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0293947	07-12-88	JP-A- 63277971	15-11-88
EP-A- 0158746	23-10-85	AU-B- 583484	04-05-89
		AU-A- 3582584	30-05-85
		CA-A- 1253422	02-05-89
		JP-A- 60185160	20-09-85
		US-A- 4775636	04-10-88